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Preparation of a Clinically Active Adrenocorticotropic Hormone (ACTH) in Good Yield from Sheep Pituitary Glands.* (19175)

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The isolation of adrenocorticotropic hormone (ACTH) from sheep and swine pituitary glands has already been described(1,2). It may be noted that the yields of the hormone prepared by these procedures are low. We wish to describe herewith a simple procedure for obtaining in much higher yield an adrenocorticotropic hormone (ACTH) preparation which is practically free from both anterior and posterior pituitary hormone contaminants, and is effective in clinical applications.

The following steps were performed at room

TABLE I. Bioassay of ACTH Fractions by the Ascorbic Acid Depletion Method.

Fraction	Dose, μg	No. of rats	Avg ascorbic acid depletion, mg/100 g adrenal	ACTH standard equivalent,* μg
Acid-acetone powder	5	10	63 ± 5†	.8
NaCl precipitate‡	5	35	126 ± 4	6.4
Final product after acid-heat treatment	2	16	111 ± 1	3.8

* These values represent ascorbic acid depleting activity in terms of standard ACTH prepared in this laboratory.

† Mean ± standard error.

‡ Obtained by NaCl precipitation between .06 and 1 saturation.

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temperature (22-24°C) except for the precipitation of acid-acetone extract which was carried out in a cold room (2-3°C).

Acid-acetone powder (AAP). One kilogram of frozen whole sheep pituitary glands was finely ground and extracted with 4.1 liters of acid-acetone solution† by vigorous stirring for one hour. The mixture was filtered and the residue was re-extracted with 2 liters of 80% acetone. After removal of the residue by filtration, the combined extracts were poured into 30 liters of cold acetone. The precipitate formed was dried in vacuum after repeated washing with acetone. The product is designated as "acid acetone powder" (AAP) and the yield from one kg of glands averages 35 g. *NaCl fractionation.* Twenty grams of AAP was next dissolved in 940 cc water and adjusted to pH 3.0. A saturated NaCl solution (60 cc) was added dropwise with constant stirring: the precipitate formed was centrifuged off and saved for the isolation of lactogenic hormone(3). The supernatant was brought to saturation with solid NaCl: the precipitate formed was separated and dissolved in 100 cc water and dialysed against running water until salt-free. The dialysed solution was frozen and dried in vacuum; yield, 3 g. *Acid-heat treatment.* The dry product (3 g) was next dissolved in 300 cc 0.2 M HCl, and the solution was kept in a boiling water bath for 60 minutes. After cooling, 1 M NaOH was added to pH 3.0. The slightly acidified solution was lyophilized; the dry solid is the final product.

From 1 kg of fresh sheep whole pituitary glands,‡ approximately 5 g§ of the final product may be obtained. Testing of the various discarded fractions by the ascorbic assay method provided evidence that the recovery of ACTH by this method is practically complete. As shown in Table I, the preparation has an ACTH potency, as estimated by the

† The acid-acetone solution was prepared by mixing 0.5 l of water, 4 l of acetone and 100 cc concentrated HCl.

‡ A similar ACTH preparation from swine pituitary glands may also be prepared by the same procedure.

§ This value was obtained by deduction of the amount of NaCl from the total solid.

TABLE II. Assay by Maintenance Test of Purified ACTH Preparation.*

Group (No. of rats)	Total dose,† mg	Body wt (range)		Avg organ wt (ranges)			
		Initial, g	Final, g	Adrenals (2), mg	Thymus, mg	Thyroids, mg	Testes, mg
Hypophysectomized controls (6)	0	52 (43-56)	60 (56-64)	6.3 (5-8)	93 (75-128)	5.6 (5-7)	84 (59-98)
Hypophysectomized treated with ACTH (7)	36	55 (46-60)	60 (53-67)	22.1 (19-24)	32 (22-68)	5 (3-8)	90 (72-119)
Normal controls (5)	0	50 (46-57)	147 (127-164)	24.2 (17-32)	324 (275-379)	13.4 (11-16)	1314 (1074-1492)

* Prepared by the procedure described in this paper.

† Male rats of the Long-Evans strain hypophysectomized at 21 days of age were used; intraperitoneal injections begun on the day of hypophysectomy and continued for a period of 21 days. The rats received two injections daily (.5 mg per inj. for the first 6 days, then 1 mg per inj. for 15 days); controls were inj. with saline. (Unpublished data of Ulrich, Reinhardt and Li, 1951).

TABLE III. Effect of Purified ACTH Preparation* Upon Circulating Eosinophiles, Fasting Blood Sugar, Urinary 17-Ketosteroids, Urinary Sodium, and Body Weight.

		Patient L. G.			Patient R. D.		
		Pre-treatment	ACTH	Post-treatment	Pre-treatment	ACTH	Post-treatment
Circulating eosinophiles, per cu mm	Mean	127	16	83	98	8	120
	Range	(93-160)	(12-22)	(50-103)	(78-115)	(7-9)	(93-135)
Fasting blood sugar, mg per 100 cc	Mean	77	92	78	68	87	58
	Range	(68-84)	(79-101)	(75-82)	(65-69)	(73-95)	(57-60)
Urinary 17-ketosteroids, mg per 24 hr	Mean	5.3	7.8	5.4	5.6	10.5	4.6
	Range	(2.3-7.3)	(6.1-9.1)	(4.6-6)	(3.9-7.6)	(6.8-14.4)	(3.6-5.2)
Urinary Na^+ mEq. per 24 hr	Mean	105	33	158	76	37	162
	Range	(82-133)	(13-80)	(34-300)	(48-100)	(14-72)	(49-250)
Body wt, kg		46.3-46.9 Max. range for 6 days	49.2 After 7 days Rx	46.8 3 days after withdrawal	73-73.4 Max. range for 6 days	75.6 After 6 days Rx	71.5 6 days after withdrawal

* Prepared by the procedure described in this paper.

ascorbic acid depletion method(4), equivalent to that of the hormone isolated by the procedure previously described(1). The product also exhibits activity in maintaining the adrenal weight of hypophysectomized rats, as is evident from the data in Table II. From the same table, it is clear that the preparation is free from gonad-stimulating, growth-promoting and thyrotropic activities as determined by target organ and body weight changes. The preparation is also substantially free from contaminating antidiuretic and pressor principles(5). No tests were made for lactogenic or oxytocic activity.

The therapeutic and metabolic effects of the preparation were investigated on 4 patients with active rheumatoid arthritis. The subjects were maintained on a carefully controlled metabolic balance regimen during treatment periods of from 6 to 12 days and appropriate control periods. The dietary intake of total calories, carbohydrate, fat, protein, and minerals was constant from day to day. Twenty-four hour urine collections were obtained, and stools were pooled for 6-day periods. Every 6 hours during treatment periods each patient received an intramuscular injection of this ACTH preparation in doses equivalent to 25 mg LA-I-A (Armour Standard) as assayed by the ascorbic-acid depletion method(4). The results which were obtained followed the patterns which have become familiar in the use of other ACTH preparations(6). In every case there occurred a significant remission of heat, pain, tenderness, limitation of motion, and soft-tissue swelling of involved joints. Those patients in whom body temperatures had been elevated became afebrile. All patients experienced an increased sense of well-being. This clinical improvement became apparent within 24 hours after treatment was started, and relapse began within 24 hours of the time treatment was withdrawn.

The clinical evidence that the ACTH preparation obtained by the procedure herein described contained a potent adrenocorticotropic principle was confirmed by laboratory tests. Treatment periods were characterized by sodium retention, water retention, weight gain, eosinopenia, an increase in urinary 17-ketosteroids, and a return of erythrocyte sedimentation rates toward normal. When the treatment was stopped these effects were promptly and completely reversed. Data from two of the cases which illustrate some of these points are presented in Table III. In every case, although over-all potassium balances were unaffected by treatment, a marked decrease in urinary excretion of both potassium and phosphate occurred during the first 24 hours following withdrawal of the treatment. No untoward effects were encountered. There was no evidence of local tissue irritation or of a systemic reaction to pyrogens. Treatment periods were not of sufficient duration to show whether prolonged use of this preparation would result in undesirable metabolic effects. Presumably it would not differ from other ACTH preparations in this respect.

Summary. A method is described for the preparation in high yield, from whole sheep pituitary glands, of purified ACTH, substantially free from growth, gonadotropic, thyrotropic, antidiuretic and pressor activities. Clinical and laboratory evidence indicates that ACTH prepared by this method is therapeutically and metabolically effective in patients with rheumatoid arthritis.

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Chromatographic Behavior of Adrenocorticotropic Peptides in Diatomaceous Earth and Starch Column.* (19176)

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In a recent paper(1), we described the purification of adrenocorticotropically active peptides (ACTH peptides) in paper partition chromatography, displacement development analysis and carrier displacement chromatography. The present paper concerns the results obtained from starch and diatomaceous earth chromatographic columns. A preliminary account on the purification of ACTH peptides in starch has appeared(2).

Experimental. The ACTH peptide mixture was prepared from the pepsin digest of the sheep protein hormone by the method previously described(2,3). The adrenocorticotropic activity was estimated by the ascorbic acid depletion in the adrenals of hypophysectomized male rats(4).

The potato starch and diatomaceous earth‡ were commercial products. The columns were prepared and operated according to the procedure described by Stein and Moore(5,6). The effluent fractions (0.5 cc) were analyzed by the ninhydrin colorimetric method(7); alternate tubes were selectively pooled and dried out and the nitrogen content was determined by the micro-Kjeldahl technic.§ The column size was 0.9 x 30 cm and the rate of flow was maintained by a pressure head of 15 cm Hg. Some of the developing solvents were those suggested by Stein and Moore (5,6) for the separation of amino acids.

Results. Three developing solvents were employed for the *diatomaceous earth* column: Solvent A, ethanol: 0.9% NaCl = 1:1; Solvent B, butanol: glacial acetic acid: water = 63:10:25, and Solvent C, benzyl alcohol: butanol: water = 1:1:0.288 with an addition of 0.5% thiodiglycol. It may be noted in Table I that a large amount of ninhydrin-reacting material appears in Fractions No. 42-62, comprising 70% of the material applied in the column when Solvent A was used as the eluent. There are some small peaks after Fraction No. 115, indicating that a large number of components is present in the original peptide mixture. A recovery of 97% of the nitrogen was achieved. Using Solvent B as the developing solvent, a large

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† Cutter Laboratories Predoctorate Fellow, 1950-51.

‡ The diatomaceous earth, Dicalite 223-T, was a special acid washed material and obtained through the courtesy of Dr. G. W. Breger, Great Lake Carbon Corp., Walteria, Calif.

§ We wish to thank Harold Papkoff for the nitrogen determinations.

TABLE I. Nitrogen Distribution of ACTH Peptides in Diatomaceous Earth and Starch Chromatography.

Chromatography	Solvent	Fraction No.	Nitrogen Content, μg	Nitrogen Recovery, %
Diatomaceous earth	A	42-62	441	97
		64-249	188	
	B	23-40	743	100
		42-141	340	
		155-200	304	
	C	26-42	202	100
		44-64	187	
		66-91	468	
		93-299	828	
Starch	D	26-44	416	91
		46-220	174	
	E	6-20	1298	100
		22-37	246	
	A	9-20 (Fraction A)	872	
		23-34 (Fraction B)	55	71

Solvent A: Ethanol: .9% NaCl = 1:1. B: Butanol: glacial acetic acid: water = 63:10:25. C: Benzyl alcohol: butanol: water = 1:1:.288. D: Butanol: propanol: .1 M HCl = 1:2:1. E: n-Propanol: .5 M HCl = 1:1.

amount of nitrogen was again recovered in the first peak (Table I). The second peak, appearing at the end of the run, is particularly interesting, as it contains 22% of the original nitrogen and must represent a distinct fraction in comparison with the peak in front. Solvent C resolves the peptide mixture into a number of small peaks and the pattern is very different from that obtained by the other two solvents. The fractionation here is only partial, due to the failure of any of the peaks to return completely to the base line. All the nitrogen was recovered in the effluent (Table I).

Three developing solvents were employed for the *starch* column: Solvent D, butanol: propanol: 0.1 N HCl = 1:2:1; Solvent E, n-propanol: 0.5 HCl = 1:1; and Solvent A. When Solvent D serves as the developing solvent, a large ninhydrin-reacting peak occurs in the region just before the appearance of leucine-isoleucine in the chromatogram of Stein and Moore(5). This large peak contains 70% of the nitrogen applied initially to the column (Table I). There are only 3 small peaks after Fraction No. 60.

Two distinct peaks were obtained in Solvent E. The data in Table I indicate that 84% of the nitrogen was found in the first fraction, in contrast to 16% in the second. The area of the 2 peaks is of interest, since it shows that the amount of ninhydrin-reacting material is almost the same for these two fractions. Hence, the average length of peptide chain in the two fractions must be different. As shown in Fig. 1, Solvent A resolved the peptide mixture into 2 large peaks. In contrast to Solvent E, the nitrogen recovery in this case was only 70%; no more nitrogen appeared after Fraction No. 50. Biological assay of the 2 peaks (Fractions A and B) showed that both fractions have adrenal-stimulating activity. Judging by assay data summarized in Table II, the adrenocorticotropic potency of Fractions A and B is similar.

Discussion. Starch columns chromatography has been employed by Borsook *et al.* (8,9) for the isolation of a peptide fraction

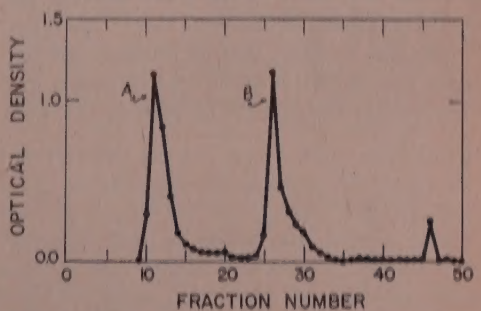

FIG. 1. Chromatogram of 10 mg ACTH peptides in starch column; solvent, ethanol: .9% NaCl = 1:1.

TABLE II. Biologic Activity Distribution of ACTH Peptides in Starch Chromatogram.*

Fraction	No. of animals	Ascorbic acid depletion/100 g adrenal,† mg	ACTH standard equivalent,‡ μg
A	21	126 ± 28§	6.4
B	10	129 ± 38§	7

* Ethanol: .9% NaCl = 1:1 used as the eluent, see Fig. 1.

† 1 μg N per 100 g body wt was inj. intrav. for assay.

‡ Estimated from a stand. assay curve, see (1).

§ Mean ± stand. dev.

from liver homogenate of various species. Their Peptide A fraction was eluted from the starch column by Solvent D a short time before the appearance of leucine. This result resembles the pattern obtained with the ACTH peptides. Since it is now clear that the peptic digest of ACTH protein is a very complex mixture(1), the occurrence of a single large fraction in the starch column cannot be taken to indicate that the fraction consists of a single peptide.

It is of interest to note that 2 distinct fractions appear in Solvents A and E in the starch column, both of which fractions are biologically active. This may suggest that more than one adrenocorticotropically active peptide occurs in the peptide mixture. Earlier studies(1) also support this view.

It should be noted that the chromatographic patterns of the ACTH peptides in starch and in diatomaceous earth columns obtained by Solvents A and D are similar. On the other hand, when the same eluting solvent is used, the behavior of the peptide mixture in these columns is quite different: One gives rise to only one large peak at the front, whereas the starch is capable of resolving into 2 fractions. It may also be noted that all nitrogen initially applied to the diatomaceous earth column was successfully removed by Solvent A, but only 70% of nitrogen recovery was achieved in the starch. If both starch and diatomaceous earth serve only as inert support for the true partition chromatography, one would expect to obtain the same chromatographic patterns in the 2 columns which have been developed by the same eluent. Hence, the results indicate that difference in degree of adsorption must play an important role in these chromatographic columns.

Summary. The ACTH peptides obtained from the pepsin digest of sheep protein hormone has been analysed chromatographically in starch and diatomaceous earth columns using various solvents as eluent. Results indicate the presence of many components in the peptide mixture. The presence of two ACTH active components in the peptide mixture has been suggested by starch column chromatography, using ethanol:0.9% NaCl as eluent.

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Action of Propylene Glycol upon Experimental Tuberculosis in Guinea Pigs. (19177)

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In researches on substances inhibiting growth of the tubercle bacillus, attention has been called to the glycols (propylene, dipropylene, ethylene, diethylene, triethylene, 1-3 butylene, 1-4 butylene; 2-amino-metil-1, 3-propandiol, polypropylene and other related compounds).

It has been demonstrated that 5-7% propylene glycol (PG) partially inhibits the *in vitro* growth of *Mycobacterium tbc., var. Hom.*, and that at 8-10% the inhibition is complete(2). Higher concentrations are required to inhibit the growth of bovine and avian strains; therefore PG is more active *in vitro* against the human than against bovine or avian strains. It is also less active against acid fast nonpathogenic mycobacteria. PG is equally inhibitory to streptomycin susceptible and streptomycin resistant strains(21). The activity of PG was therefore tested *in vivo*.

The toxological effects of PG are given by the following investigators: Hunt(12), Seidenfeld and Hanzlik(24), Brown(4), Braun and Cartland(3), Holck(11), Lehman and Newman(17-19), Van Winkle and Newman(27), Weatherby and Haag(28), Aiazzi-Mancini (1), Hanzlik *et al.*(9-10), Dumez(6), Latven and Molitor(14), Laug *et al.*(15), Kesten *et al.*(13), Newman *et al.*(20), Smith(25), Van Winkle(26), Bucciardi(5), Morris *et al.* (18), Launoy(16), Fabre and Rousseau(7), Randolph and Mallery(22), Whitlock *et al.* (29), Robertson *et al.*(23). Preliminary toxicological tests were made on guinea pigs and rabbits. These confirmed the data of previous workers.

In these experiments on the efficacy of PG in experimental tuberculosis, guinea pigs were treated with PG administered orally, parenterally (subcutaneously) and by aerosols. Oral administration has no antituberculous effect. Only experiments on guinea pigs in which the PG was administered subcutaneously are reported here.

Experimental. Guinea pigs were infected by subinguinal inoculation with 0.1 mg (dry weight) of an H. virulent strain (H. 49, Ist. "Principi di Piemonte"). They were divided into several groups:

Group		No. of animals
I	Guinea pigs infected and killed every 5th day	60
II	Guinea pigs infected and treated with PG. This group was subdivided into:	
	(a) those in whom treatment was begun immediately after infection	60
	(b) those in whom treatment was begun 8 days after infection	60
	(c) those in whom treatment was begun 21 days after infection	60
III	Control infected guinea pigs that died spontaneously	70
IV	Infected guinea pigs treated with PG that died spontaneously	70

Treatment. Preliminary tests were made in experimentally infected guinea pigs with various concentrations of PG (5% to 30%). We used a racemic compound, m.w. 76.09; b.p. 188.2. A 20% saline solution was found optimal, but lesser concentrations also have a therapeutic action. The guinea pigs were injected subcutaneously twice daily with 2 cc of 20% dilution of PG. This dose is well tolerated for a prolonged time, as demonstrated in another group of 30 guinea pigs, given PG only. To investigate the macroscopic tuberculous alterations in the several organs the guinea pigs of Group I were killed each 5th day. Several infected guinea pigs which died earlier were not included. The guinea pigs of Group II were infected and treated with PG at various times after infection: in subgroup (a) immediately after infection, in order to note the effect of PG upon the formation of earlier alterations; in (b) after 8 days and in (c) after 21 days. The treated guinea pigs, like the controls, were killed each 5th day and the macroscopic tuberculous alterations in the different organs

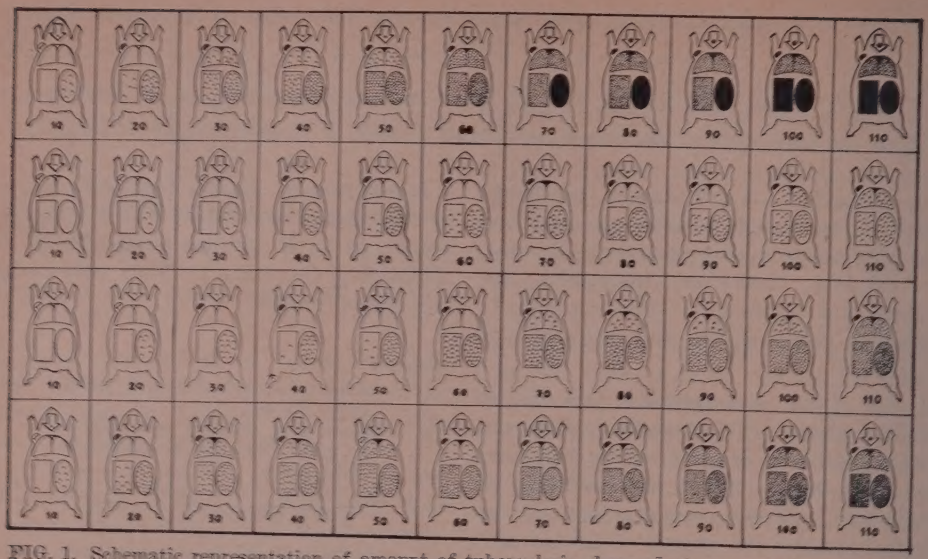

FIG. 1. Schematic representation of amount of tuberculosis observed at necropsy in untreated guinea pigs (first line), and in treated animals, in which the daily treatment is started immediately after infection (second line); 8 days after infection (third line), and 21 days after infection (fourth line).

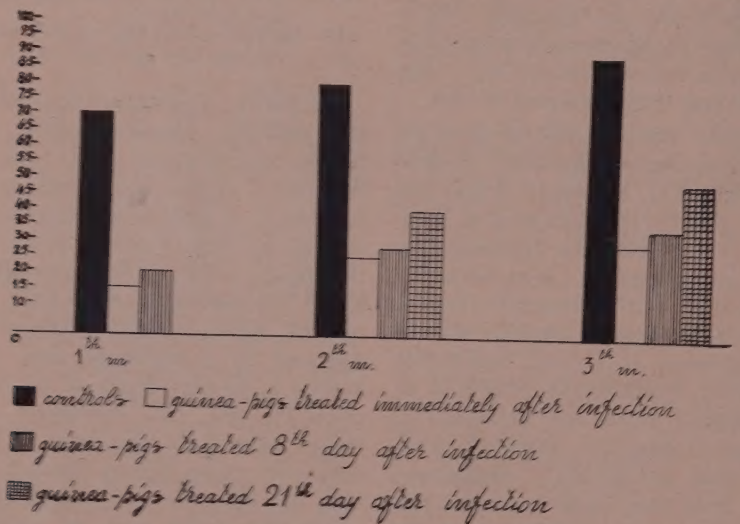

FIG. 2. Index of tuberculization at end of each month.

noted. These experiments were continued until the 110th day after inoculation. Guinea pigs of Groups III and IV were infected with the same bacillary suspensions. Group III served as untreated controls, Group IV was treated daily with the optimal dose of PG, beginning with the 10th day after infection.

Results. The degree of tuberculosis, observed macroscopically, at necropsy, every 10th day, in treated and untreated guinea pigs is shown in Fig. 1. Changes are indicated according to the scheme of Feldman and Hinshaw(8). Comparison of the macroscopic changes in controls and in treated guinea pigs shows that the treatment had a favorable effect upon the intensity and extent of the

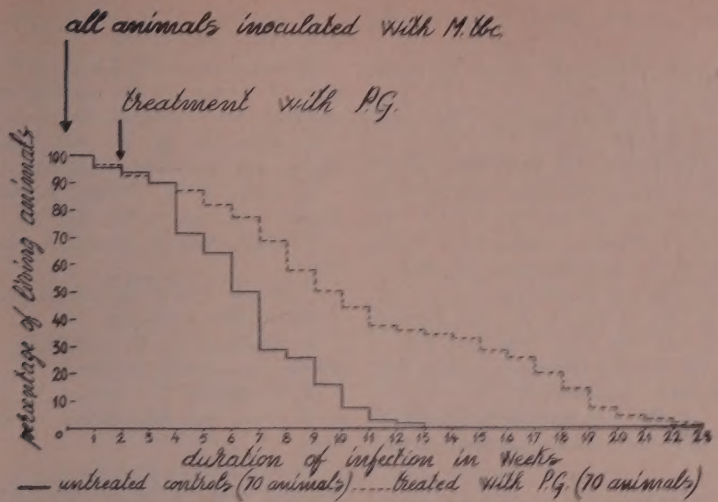

FIG. 3. Comparative survival times of guinea pigs treated with propylene glycol and in untreated controls.

alterations in the various organs. Histological studies confirmed the macroscopic observations. From the extent and character of the macroscopic tuberculous alterations in several organs of guinea pigs of Group I and of various subgroups of Group II, the index of tuberculization was calculated at the end of each month (Fig. 2).

The reduction of the tuberculization index occurred in the first and second subgroups (a and b), *i.e.* in animals that had been exposed to treatment immediately or 8 days after inoculation. In the first month the index is 15-20% against the 70% for the controls; at the end of the second month the indices are: 25%, 28%, and 40%, respectively, for each subgroup against 80% for the controls. At the end of the third month the index is 30%, 35%, 50%, respectively, for each subgroup against 90% for the controls. After a longer time (this experiment was continued to the 110th day) these differences were reduced, but still remain large. Fig. 3 shows the comparative survival times in weeks, of treated and untreated tuberculous guinea pigs (Group III and IV), which died spontaneously. While all controls died by the 13th week, several treated guinea pigs were alive and the last one died in the 23rd week.

Conclusions. In experimentally infected guinea pigs, subcutaneous treatment with PG inhibits extensive tuberculous alterations and prolongs the survival time. Therefore, propylene glycol exerts a favorable action *in vivo*. Its *in vivo* suppression of tuberculosis is equal to that of other substances with *in vitro* bacteriostatic properties. The antituberculous power of propylene glycol *in vivo* is greater when the treatment begins immediately after infection.

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Detection of Antibodies by Polyvinylpyrrolidone* II. Comparison of Sensitivity of Antihuman Globulin and PVP. (19178)

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This work, comparing the sensitivity of the Coomb's test and PVP titration to detect anti Rh antibody, is a natural outgrowth of our original publication concerning polyvinylpyrrolidone as a suitable antiserum diluent(1). Most serologists now concede that the Coomb's test excels other methods of incomplete antibody detection both in sharpness of reaction and sensitivity(2-6). Therefore, if PVP technics compare favorably with that of employing anti-human globulin for detection of antibodies, further expansion of the uses of PVP might be justified.

Materials. (1) Antisera—anti Rho′ (CD) serum was used throughout the titrations. All of this reagent came from the same lot and has an albumin titer of approximately 1:128. The saline agglutinin titer was almost nil. (2) Red blood cell suspensions were obtained from a single donor whose type was O Rho′ (CDe). The cells were drawn less than 5 days prior to use. 20% bovine albumin (Armour & Co.) in 0.9% saline was used as a diluent. (3) 10% Polyvinylpyrrolidone (PVP) was prepared as previously described (1) buffered to pH 6.9-7.0. (4) Anti-human globulin was obtained from a commercial source (Ortho) having passed the requirement for U. S. license. (5) Albumin 20% was prepared from 30% bovine albumin fraction II (Armour & Co.).

Methods. Titrations were carried out, using the serial dilution tube (size 75 x 10 mm) technic. In each titration, 0.2 ml of anti Rho′ blocking serum was mixed with an equal quantity of diluent, as indicated in Table I, and 0.2 ml was transferred to the succeeding tube, which also contained an equal amount of diluent. The double dilution of serum was followed by the addition of 0.2 ml of a 4% fresh washed suspension of Rho′ (CDe) red blood cells in saline or 20% albumin as indicated by the table. Mixing and incubation of tubes at 37°C water bath for one hour was

* Polyvinylpyrrolidone kindly furnished by General Aniline & Film Corp., Easton, Pa.

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TABLE 1. Comparison of Sensitivity of Antibody Detection Using Indirect Coomb's Technic. Anti Rho' (CD) blocking serum diluted as follows:

Titer	Section 1, saline diluted serum: Cells in saline Tested with saline	Cells in saline Tested with albumin	Cells in saline Tested with PVP	Cells in saline Tested with antihuman globulin	Section 2, 20% albumin diluted serum: Cells in albumin Washed with saline. Tested with saline	Cells in albumin Washed with saline. Tested with albumin	Cells in albumin Washed with saline. Tested with PVP	Cells in albumin Washed with saline. Tested with antihuman globulin	Section 3, 10% PVP diluted serum: Cells in saline Tested with saline	Cells in saline Tested with albumin	Cells in saline Tested with PVP	Cells in saline Tested with antihuman globulin	Alternate technic. Serum dilution. Cells in saline Immed. centrif. 1 min
4	+		+	+	+		+	+	+		+	+	+
8	+		+	+	+		+	+	+		+	+	+
16	+		+	+	+		+	+	+		+	+	+
32	+		+	+	+		+	+	+		+	+	+
64	+		+	+	+		+	+	+		+	+	+
128	+		+	+	+		+	+	+		+	+	+
256	+		+	+	+		+	+	+		+	+	+
512	+		+	+	+		+	+	+		+	+	+
1024	+		+	+	+		+	+	+		+	+	+
2048	+		+	+	+		+	+	+		+	+	+

Controls: 1, Rh neg (cde) cells in serum (CD)—Coomb's tested—neg. 2, Rh neg (cde) cells in PVP—PVP tested—neg. 3, Rh pos cells (CDe) in albumin—Coomb's tested—neg. 4, Rh pos cells (CDe) in saline—Coomb's tested—neg.

followed by repeated thorough washing (3x) of the cells with 0.9% saline. Finally each series of tubes received 2 drops of either saline, albumin, 10% PVP or antihuman globulin, as indicated in the table. Re-incubation for one hour and centrifugation 1 minute at 1500 r.p.m. followed by gently tapping the tube for the customary reading, completed the test.

Alternate PVP technic. A rapid tube technic utilizing PVP was developed for comparison with the indirect Coomb's technic. The same serum and cell suspension was used as follows: the serum was titered by the same double dilution serial technic, using 10% PVP as diluent. Each tube then received an equal volume (0.2 ml.) of unwashed 4% saline suspended red cell antigen. The tubes were shaken to assure mixture and centrifuged immediately for 1 minute at 1500 r.p.m. Readings were made promptly after centrifugation. The entire test could be performed in less than 10 minutes.

Results. The table of results is expressed in 3 sections for simplicity and clarity. Section 1 shows the results of the quantitative Coomb's technic using saline as antiserum diluent. Each vertical column represents a different substance added at the end as a "*testing*" reagent. Section 2 represents antiserum diluted with albumin and in Section 3, PVP was used as diluent.

Inspection of the table will reveal the following facts:

Section 1—Cells "coated" by antisera diluted in saline react poorly in saline, fair in albumin, fair in PVP and good in antihuman globulin.

Section 2—Cells "coated" by antisera diluted in albumin react poorly in saline, fair in albumin, good in both PVP and anti-human globulin.

Section 3—Cells "coated" by antisera diluted in PVP react poorly in saline and albumin, good in both PVP and anti-human globulin.

Discussion. The sensitivity range of detecting antibody "coated" erythrocytes using PVP compares favorably with antihuman globulin, though slightly less potent. When the cells are incubated in saline medium the

action of PVP is poor, but in either albumin or PVP medium the sensitivity is good. The alternate simple technic given in Section 3 of the Table appears to give excellent results and avoids the cumbersome washing and incubation which is necessary in the indirect Coomb's test.

Although our case studies of sensitized mothers and infants carrying anti Rh antibodies is incomplete, the indication is that PVP detects these antibodies in rather high dilution even though albumin technics are negative or weakly positive. In addition, we have revealed an abnormal antibody to be present in the serum of a young woman who has been clinically diagnosed as lupus erythematosus. PVP revealed this antibody in equal titer to anti-human globulin, whereas saline and albumin titrations were completely negative. A similar case is reported by Callender(7). It appears that polyvinylpyrrilodone offers a simple, economical and sensitive method of Rh antibody detection.

Summary. (1) Using the indirect Coomb's technic we have found that 10% PVP offers results comparable to anti-human globulin when dilutions are carried out in albumin or PVP. (2) An alternate rapid PVP tube test (of equal sensitivity to antihuman globulin) is described. (3) In certain "collagen" or hypersensitivity diseases, PVP may detect an agglutinating antibody on homologous erythrocytes.

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Effects of X Irradiation on Renal Function in Newly-Hatched Chicks. (19179)

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The effects of X irradiation on young birds is dependent on dose rate as well as on total dose(1,2). Chicks and ducklings given 1000 r within a period of 30 minutes showed 75 to 80% mortality in the initial period (24 to 48 hours) after irradiation. When the same total dose was delivered over a 3-hour period, few deaths occurred until 5 to 7 days after exposure. Birds that died in the initial period showed necrosis of the renal tubules and accumulation of urate crystals in and around the tubules. A few individuals showed a urate polyserositis, in which a thin layer of urate crystals covered all serous membranes. Uric acid is the principal end product of protein metabolism in birds, and its precipitation throughout the body indicated a rapidly developing azotemia. This condition may be assumed to result either from renal failure or from an increased rate of nitrogen metabolism without a corresponding increase in excretion. The experiments reported here were concerned with the effects of X irradiation on renal function in the newly-hatched chick, as determined by estimations of blood urate levels and excretion of uric acid and phenol red.

Materials. White leghorn chicks, weighing 35 to 45 g, were treated 2 to 4 days after hatching. The irradiation dose was 1000 r, delivered at 6 r or at 43 r per minute. The conditions of irradiation were: 200 kv, 15 ma, 0.5 mm Cu and 3.0 mm Bakelite filters. Variation in dose rate was obtained by vary-

TABLE I. Effect of X Irradiation on Excretion of Uric Acid in Newly Hatched Chicks.

	Control	1000 r X radiation		
Exposure	—	6 r/min or 43 r/min*	43 r/min	
No. animals	10	6	5	11
Survival after X ray (hr)	—	24	5–6	3–4
Time after X ray (hr)	mg uric acid			
0-1	5.88	6.74	5.34	3.79
1-2	4.46	4.31	2.91	.67
2-3	4.72	5.66	1.65	.21
3-4	4.17	4.06	.38	.08
4-5	3.88	5.76	.02	
5-6	—	—	0	
5-24	117.8	150.2		
Mean (mg/hr)	5.59	7.43		

* Excretion of 24 hr survivors not influenced by exposure rate.

ing the target field distance. Blood urate determinations were made on heart blood, using the method described by Brown (3). For estimations of rate of urate excretion, accumulated material was removed from the cloaca at intervals of 0.5 hour or more and the quantity of uric acid present was determined colorimetrically. The amount of phenol red in excreta was estimated at the same intervals for 2 hours after intraperitoneal injection of 0.2 mg of the dye. All quantitative determinations were made photoelectrically.

Results. Determinations of blood urate were made at 0.5, 1, 3, and 5 hours after irradiation. At 1 hour after irradiation, the mean blood urate in 15 animals (10.8 mg %) did not differ from the mean established in 26 control animals (8.2 mg %). A marked increase (27.1 mg %, 17 animals) occurred at 3 hours after exposure at 43 r/min and a further increase (33.0 mg %, 17 animals) at 5 hours. Values were still higher (60.0 mg %) in six animals moribund at 3 to 5 hours. After exposure at 6 r/min, only a small increase (10.2 and 18.8 mg %) occurred at 3 and 5 hours. Visible precipitated urates in the kidneys were present in all animals that had high blood urate levels.

Table I shows urate excretion in control and in irradiated chicks, grouped according to survival time after exposure. Following irradiation, urate excretion continued at a normal rate for at least 1 hour. Beginning inactivity was accompanied by a decrease in urate excretion, and an essentially complete anuria preceded death. The rate of urate excretion was normal in irradiated birds that survived more than 24 hours, whether the exposure rate was 6 r or 43 r per minute. At death, the deficit in excreted urate ranged from 20 to 60 mg per 100 g body weight. If the urate space is assumed to equal the extracellular body water (approximately 20% of body weight), then the urate deficit corresponded to blood levels of 90 to 290 mg % in excess of normal. These calculated values are much higher than observed blood values, which ranged from 30 to 60 mg % in excess of normal, and indicate that some urate was present in the solid phase. This condition had been observed in numerous individuals sacrificed in a moribund condition. Concentrations of blood urate tended to be slightly higher than normal in irradiated chicks surviving at 24 hours, and a few birds sacrificed 24 hours after exposure showed accumulated urate in the kidneys.

The effect of X-ray exposure on renal tubular function was determined by measurement of phenol red excretion. In 2- to 4-day untreated birds, one third of the injected dose was excreted in the first 30 minutes and a total of two thirds was eliminated within 2 hours. On the basis of amount of phenol red excreted, chicks irradiated at 43 r per minute fell roughly into two groups: those that died within 10 hours after exposure, which excreted only a small amount within the first two hours: and those that lived 24 hours or more after irradiation, in which the excretion rate was essentially

TABLE II. Effect of X Irradiation (1000 r, 43 r/Min) on Excretion of Phenol Red.

Hr between irrad. and inj. of .2 mg phenol red	No. of animals	Survival time after irradiation	% of inj. dose excreted in 2 hr
Not irrad.	17	—	63.5
.5	4	5.5–8.5 hr	28.2
	2	1–3 days	76.9
1	3	5.5–6 hr	28.3
	1	3 days	79.4
2	9	4.5–8 hr	9.3
	2	1–2 days	39.9

normal during the first few hours (Table II). Exposure at a dose rate of 6 r per minute had little effect on phenol red excretion, and results resembled those obtained on 24-hour survivors exposed at 43 r per minute.

Determinations of the simultaneous excretion of urate and phenol red were made following exposure at 6 r and 43 r per minute. Birds that received phenol red 1 hour after a low rate exposure excreted urate and phenol red at a normal rate. When phenol red was injected 1 hour after a high rate exposure, excretion of both substances was essentially normal in 24-hour survivors, but was low or unmeasurable in birds that died within 10 hours after exposure. In some individuals, phenol red excretion was completely inhibited during a period when minute quantities of urate were collected, but in no case was the dye excreted in the absence of urate. These data indicate a similar mechanism of excretion for the 2 substances or the simultaneous failure of 2 different mechanisms.

Discussion. The radiation-induced renal lesions, primarily necrosis of proximal convoluted tubules, as well as the effects on urate and phenol red excretion, indicate that renal failure is the immediate cause of the early deaths that follow high rate exposures. Mammals with complete renal failure may live for several days, but in birds an obstructive uremia (produced by bilateral ligation of the ureters) results in death within 12 to 24 hours(4-6). Blood urate levels, 5 hours after ureteral obstruction, averaged 50 mg % above normal, and at death the birds commonly showed a severe urate polyserositis. The rapidly developing toxemia may not be only the result of toxicity of the accumulated urate. Lithium urate solution administered intraperitoneally in quantities sufficient to raise the extracellular urate concentration 200 mg % above normal was rapidly excreted and produced no visible clinical effect nor any change in radiation sensitivity in newly-hatched chicks(6). It is probable that lithium protected these birds against death through its effect on urate solubility. In addition, other accumulated byproducts of metabolic processes may contribute to the toxemia.

Comparison of urate deficit at death with the estimated total amount present in the blood and precipitated in kidneys and on serous linings indicated that ionizing radiations did not interfere with the synthesis of urate. In chicks, the formation of urate from ammonia is probably carried out entirely by enzymes present in the liver(7). It would be of interest to study acute radiation effects in the pigeon, in which the final conversion of hypoxanthine to uric acid takes place, at least in part, in the kidney.

Shannon(8) has shown that the primary mechanism for urate excretion in the chicken is by means of the renal tubules. The necrosis of renal tubular cells following high rate exposures indicates a possible widespread inactivation of mechanisms essential for urate excretion, and it would appear that in birds the tubular epithelium has a greater radiation sensitivity than it has in mammals. Other factors can also influence renal function, *e.g.*, (1) maintenance of a minimal blood pressure necessary for glomerular filtration, (2) activity of enzyme systems in the cells of the secretory tubules, and (3) extrarenal factors, including the adrenal cortex and the pituitary-adrenal relationship. X irradiation has been shown to produce a sharp fall in blood pressure in rabbits(9) but its effect on the blood pressure of the irradiated chick remains to be investigated.

These experiments indicate that, in the bird, death in the initial period after irradiation is preceded by acute renal failure. Further investigations will be concerned with determination of the relative importance of (1) direct radiation damage to the kidney and (2)

indirect effects upon renal function produced by generalized physiological disturbances in the organism.

Summary. Young chicks were exposed to 1000 r X radiation at dose rates of 6 or 43 r per minute. Determinations of excretion rate of urate and phenol red and of blood urate levels indicated that death in the initial period (within 8 to 24 hours) following high rate exposures was preceded by renal failure and a rapidly developing azotemia. Individuals that survived 24 hours or more after exposure, whatever the dose rate, continued to excrete at a normal rate. Phenol red retention invariably accompanied failure of urate excretory function.

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Effect of Cortisone on Anaphylactic Response of Guinea Pig Ileum. (19180)

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A diminished antibody production(1), interference with antigen-antibody reaction(2), and altered reactivity of tissues(2) have been considered as possible mechanisms by which ACTH and cortisone influence certain hypersensitive states. The anaphylactic response of the isolated guinea pig ileum (Dale-Schultz Phenomenon) appeared to be a simple method of evaluating the importance of these postulated modes of action of cortisone on hypersensitivity of the Arthus type. In contrast to the intact guinea pig, the response of the isolated ileum is more easily standardized, and therefore tests of significance can be made with fewer animals. Other investigators have reported the inefficacy of cortisone treatment on guinea pig anaphylaxis(3,4). It is not known whether this is a species difference or a matter of dosage, since cortisone has been claimed to be effective against anaphylaxis in the mouse(5), rabbit(6), and the anaphylactoid reaction in the rat(7). Accordingly, experiments were performed on the isolated sensitized guinea pig ileum to study the effect of prolonged administration of cortisone acetate on (1) the level of tissue antibody, (2) the reactivity of tissue to histamine, and (3) the effect of cortisone acetate *in vitro* on the antigen-antibody reaction. The effects of sodium salicylate and of ethyl alcohol on the latter reaction were also investigated.

Method. Twelve virgin, female guinea pigs weighing between 260-350 g were divided at random into 4 groups of 3 animals each. Each group was injected intraperitoneally with 0.5 ml of undiluted horse serum, without preservative. Beginning on the day of sensitization and for a period of 20 days, one guinea pig in each group was injected subcutaneously with 5 mg/kg of a saline suspension of cortisone acetate, a second guinea pig with 1.0 ml/kg of 0.9% saline, and the third guinea pig in each group served as the untreated control. The saline suspension of cortisone acetate was prepared by dissolving the material in warm acetone, reagent grade, adding saline, and then removing most of the acetone by evacuation. The suspension was diluted so that each milliliter contained 5 mg of cortisone acetate. On the 20th day, all animals in

each group were injected with 0.1 ml of undiluted horse serum and 0.9% saline intradermally, and the sites of injection examined 24 hours later. No attempt was made to grade the Arthus reaction. The animals were sacrificed on the 21st day by a quick blow on the head. A section of ileum was immediately removed, washed free of blood with warm Tyrode's solution and duplicate strips about 4 cm in length were mounted in two chambers of 100 ml capacity in a constant temperature bath thermostatically maintained at 38°C. The tissues were bathed in Tyrode's solution continuously oxygenated with 100% O_2. The sections of ileum were run in parallel and the responses recorded kymographically. The experimental procedure was as follows: challenge by the addition of a standard dose of 10 γ histamine diphosphate, wash out; challenge by horse serum in a final chamber concentration of 1:2000, wash out; challenge by horse serum 1:2000, wash out; and finally challenge by the second addition of histamine diphosphate. The concentrations of cortisone acetate used in the *in vitro* experiments were prepared from a stock solution of 4 mg/ml in absolute ethyl alcohol by dilution with Tyrode's solution. Control concentrations of ethyl alcohol were prepared in a similar manner and expressed as the final concentration in the tissue chamber. The amounts of sodium salicylate (Merck) tested were added to the chamber in a volume of 0.5 ml Tyrode's solution. The experimental procedure for the *in vitro* studies was similar to that described previously, with the exception that an equilibration period of 5 or 10 minutes was allowed between the addition of the drug under test and the first challenge by horse serum 1:2000. In order to test the effect of the drug on histamine response, a wash out followed only the first addition of histamine diphosphate. In all experiments control sections of ileum from the same guinea pig were run in parallel. The guinea pigs used in these experiments were sensitized for a period of 21-30 days. All animals were housed in individual cages in air conditioned animal quarters (76°F) and maintained on Rockland stock guinea pig pellets fortified with ascorbic acid.

Results. The effect of prolonged cortisone acetate administration on the Arthus reaction, histamine reaction, anaphylactic response and the sources of variation isolated in the analysis of variance are given in Table I.

The histamine responses are expressed to the nearest millimeter and the anaphylactic response as a percentage of the mean of the histamine responses. It is evident that cortisone acetate treatment had no effect on histamine sensitivity and the reaction to a standard dose of histamine could be used as a standard to compare the anaphylactic responses. Since there was some variation between duplicate strips, the anaphylactic response was expressed as a percentage of the mean histamine response of each strip. Prolonged administration of cortisone acetate had no effect on the anaphylactic response of the isolated guinea pig ileum and did not abolish the Arthus reaction. The cortisone treated group did not gain as much weight as the two control groups but this difference was not analyzed statistically. Two guinea pigs sensitized to horse serum for 21 days and pretreated with 25 mg of cortisone acetate intraperitoneally 4½ and 6½ hours before sacrifice likewise showed no diminution in the anaphylactic response or histamine sensitivity of the isolated ileum. However, exploration of the peritoneal cavity at autopsy showed a considerable amount of unabsorbed cortisone acetate.

Table II summarizes the *in vitro* effect of cortisone acetate on the anaphylactic and histamine responses. Two sections of ileum from the same animal were run in parallel; one was controlled by the addition of ethyl alcohol. The results of cortisone acetate and ethyl alcohol in all concentrations were classified into two categories, reactors and nonreactors, arranged in a 2 x 2 contingency table and a χ^2 calculated, including the Yates correction for continuity. The χ^2, 3.74, was not significant at the P = .05 level of significance. Any inhibition of the anaphylactic response was undoubtedly due to the presence of ethyl alcohol and not cortisone acetate. This was further confirmed in six paired trials by the demonstration of an inhibitory effect

TABLE I. Effect of Cortisone Acetate Treatment on Arthus Reaction, Histamine and Anaphylactic Response of Isolated Guinea Pig Ileum.

	Cortisone			Saline			Control		
Group	Histamine, mm	Horse serum, %	Arthus	Histamine, mm	Horse serum, %	Arthus	Histamine, mm	Horse serum, %	Arthus
1	43, 45	34	+	29, 33	40	+	32, 36	45	+
	28, 33	41		38, 32	30		31, 33	51	
2	34, 39	61	+	30, 35	60	+	34, 40	46	+
	29, 30	58		36, 37	45		35, 30	75	
3	29, 40	47	+	28, 33	55	+	35, 32	54	+
	35, 40	53		31, 36	45		40, 40	40	
4	36, 40	68	+	35, 33	49	+	33, 35	71	+
	32, 37	60		34, 24	55		40, 30	60	
Wt gain, g		61			93			88	

Analysis of variance

Source of variation	D.F.*	Anaphylactic response F ratio	Anaphylactic response At. P	Histamine reaction F ratio	Histamine reaction At. P
Between treatments	2	2.03	P = .05, 3.6	1.03	P = .05, 3.6
" groups	3	7.90	P = .01, 5.1	1/F = 14.28	P = .01, 5.1
Interaction of groups × treatments	6	1/F = 1.92	P = .05, 4	1/F = 2.27	P = .05, 4
Duplicate strips	12				

* D.F. = Degrees of freedom.
Interaction of groups × treatments, and duplicate strips pooled for estimate of error.

TABLE II. Effect of Cortisone Acetate *In Vitro* (Guinea Pigs).

Guinea Pig No.	Cortisone, γ	Alcohol, %	Equilibration period, min	Anaphylactic response Cortisone	Anaphylactic response Alcohol	Histamine response Cortisone	Histamine response Alcohol
19	560	.8	10	0	+	+	−
19	560	.8	10	0	+	+	+
19	560	.8	10	0	+	+	+
19	560	.8	10	0	0	+	+
29	560	.8	10	0	0	+	+
19	400	.8	10	+	+	+	+
19	280	.4	10	+	+	+	+
30	200	.28	5	+	+	+	+
30	100	.14	10	+	+	+	−
	No. of reactors			4	7	9	9

0 = No response. $\chi^2 = 3.74$. Not significant at P = .05.

of 0.8% ethyl alcohol on the anaphylactic response of 4 out of 6 strips. All 6 control strips showed the typical response when challenged by horse serum. Neither cortisone acetate nor ethyl alcohol affected the histamine response. It was also found that those alcohol treated strips which did not respond to the first addition of horse serum, failed to respond to a second addition of horse serum even after the chamber was washed out three times with Tyrode's solution. Furthermore, the inhibitory effect of ethyl alcohol was not due to any effect on the horse serum *per se* since a solution in 0.8% ethyl alcohol elicited the typical anaphylactic response 5 minutes and 3½ hours after preparation.

The effect of sodium salicylate on the anaphylactic response is summarized in Table III. Successive additions of horse serum, 1:2000, were added to the chamber, each followed by washing with Tyrode's solution. Control strips from the same guinea pig were run in parallel. The response to the first addition of horse serum is considered 100% and rated +—.

Treatment with sodium salicylate appears to prevent complete desensitization. The data were classified into reactors and non-reactors to the second addition of antigen and a χ^2 of 2.62 calculated by means of a 2 x 2 contin-

TABLE III. *Effect of Sodium Salicylate In Vitro* (Guinea Pigs).

Guinea Pig No.	Sodium salicylate, mg	Equilibration period, min	Reaction to successive addition of antigen 1	2	3	4	(Control) Reaction to successive addition of antigen 1	2	3	4
7	25	10	4+	1+	—	—	4+	0	—	—
7	50	10	4+	2+	+	0	4+	0	—	—
17	10	10	4+	2+	0	—	4+	0	—	—
17	20	5	4+	1+	+	0	4+	+	0	—
27	20	5	4+	0	—	—	4+	0	—	—
27	10	10	4+	0	—	—	4+	0	—	—
20	100	10	4+	4+	0	—	4+	0	—	—
	No. of reactors		7	5	2	0	7	1	0	—

4+ = 100%. $\chi^2 = 2.62$, P = .10.

gency table, with a P = .10. It is possible that in a more extensive series, the difference might be significant at the P = .05 level of significance. The histamine response was not affected. There did not appear to be any clear relationship between the dose of salicylate and the response to the second addition of antigen.

Discussion. Prolonged treatment of guinea pigs with cortisone acetate did not abolish the Arthus reaction or the anaphylactic response of the isolated guinea pig ileum. If the amount of fixed tissue antibody is a true measure of antibody production and release, the process was apparently not affected by prolonged cortisone treatment. It has been questioned whether the level of tissue antibody reflects the level of circulating antibody(8). The literature is replete with contradictory statements in regard to the effect of cortisone on antibody production(9). Since the level of circulating antibody was not measured in this investigation, no definite statement can be made. It can, however, be definitely stated that prolonged cortisone acetate treatment had no effect on the level of tissue antibody, as measured by the anaphylactic response of the isolated guinea pig ileum. Hyperadrenocorticalism of endogenous origin, indicated by gross adrenal hypertrophy, induced by the injection of a stressing agent for 27 days likewise had no effect on guinea pig anaphylaxis (10). The ineffectiveness of cortisone acetate *in vitro* indicates that it had no effect on the antigen-antibody reaction in the Arthus type of hypersensitivity in the guinea pig. This is in agreement with one unpublished report (11). The reactivity of the isolated ileum to histamine was not affected by cortisone treatment *in vivo* or *in vitro*.

The abolition of the anaphylactic response by low concentrations of ethyl alcohol was not due to any effect on the antigen or the reactivity of the tissue as measured by the histamine response. It was not due to a "blockade" of the antigen-antibody reaction since repeated washing did not restore the anaphylactic response. Possibly some interference with antigen-antibody reaction or effect on tissue antibody is involved.

It has been stated that salicylates block antigen-antibody reactions(12). The incomplete desensitization of the isolated ileum after treatment with sodium salicylate would indicate some blockade of the antigen-antibody reaction. However, the effect was minimal and a large series of trials is needed to definitely establish it as fact.

Summary. Treatment of guinea pigs during the period of sensitization to horse serum with 5 mg/kg of cortisone acetate daily for 20 days did not abolish the Arthus reaction or affect the level of tissue antibody as measured by the anaphylactic response of the isolated ileum. Injection of 25 mg intraperitoneally 4½ and 6½ hours before sacrifice likewise had no effect. Cortisone acetate *in vitro* had no effect on the antigen-antibody reaction of the Arthus type. Ethyl alcohol, at a concentration of 0.8%, usually abolished the anaphylactic response. Some blockade of the antigen-antibody reaction by sodium salicylate was indicated. The histamine sensitiv-

ity of the isolated ileum was not affected by cortisone acetate treatment *in vivo* or *in vitro*.

The cortisone acetate used in these experiments was kindly supplied by Dr. S. Bernstein, Lederle Laboratories Division, American Cyanamid Co.

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Nucleic Acid Changes in Tumor-bearing Mice.* (19181)

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Reddy and Cerecedo(1) observed a marked rise in the nucleic acid content of the tissues of mice bearing transplanted Sarcoma 180, and of those with transplanted melanoma S-91. An increase in the liver desoxypentose nucleic acid (DNA) fraction of rats fed p-dimethylaminoazobenzene, long before any hepatoma developed, was found by Masayama and Yokoyama(2). These findings have been recently essentially confirmed by Price *et al.* (3). In this laboratory, similar changes were observed in the tissues of mice during gestation(4).‡

In this paper we report data showing a relationship between the rate of growth of the tumor and the changes in the nucleic acid content of certain organs in the host. We also present data on the nucleic acid, purine, and pyrimidine content of melanoma S-91 at different stages of growth.

Materials and methods. Two strains of mice, dba and the Swiss, were employed in this study. The tumors, Sarcoma 180 and malignant melanoma S-91 were obtained from The Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine. They were transplanted subcutaneously into the right pectoral region of 5- to 6-week-old mice of mixed sexes. Groups of 4, 5 or more animals were killed by decapitation at given intervals. The pooled tissues were homogenized at 0°C, and the nucleic acids extracted with hot trichloroacetic acid(5). The pentose nucleic acid (RNA) was determined according to the method of von Euler and Hahn(6), and the DNA according to that of Stumpf(7). Guanine and adenine were determined according to the methods of Hitchings(8), and of Woodhouse(9), respectively, as used in the combined procedure of Lombardo and Cerecedo (10). Uracil and cytosine were prepared in this laboratory. Thymine was generously supplied by The Fleischmann Laboratories, New York, through the courtesy of Dr. F. J. DiCarlo. The pyrimidines were estimated by means of paper chromatography. Since the

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‡ After preparing the manuscript of this paper, our attention was called to a publication of Kelly and Jones(10), who studied the turnover of desoxypentose nucleic acid in mice bearing transplants of Strong's mammary carcinoma. Their results are in harmony with our findings.

TABLE I. Nucleic Acid Changes in the Tissues of dba Mice Bearing Sarcoma 8180.

Tissue	Controls 22:5 *	1st wk 22:7 : 1.09 *	2nd wk 30:3 : 7.94 *	3rd wk (12:2 : 16.2 *
		RNA, mg/g dry wt		
Sarcoma		48.5 ± 2 †	44.3 ± 1.03†	44.3 ± 1.90†
Liver	30.2 ± .20	30 ± .46	41.5 ± 2.19	52 ± 1.79
Kidney	26.2 ± .70	22.8 ± 1.77	27.4 ± 1.41	26.2 ± .25
Lung	10.2 ± .56	11.8 ± .71	20 ± .32	16.9 ± .30
Spleen	46.4 ± .59	42.6 ± 1.56	47.1 ± .24	51.4 ± 1.05
		DNA, mg/g dry wt		
Sarcoma		35.8 ± 7.06	48.6 ± 3.96	37.5 ± 1.53
Liver	9.6 ± .44	10.3 ± .83	15.6 ± 1.99	20.6 ± .30
Kidney	21.7 ± .78	27.7 ± 1.28	32.5 ± 1.43	31.4 ± 1.10
Lung	29.8 ± .72	24.5 ± 1.90	36.2 ± 1.78	46.2 ± 6.20
Spleen	163 ± 11.16	177.2 ± 3.25	117.8 ± 9.67	155.5 ± 1.50

* The first No. in parenthesis indicates No. of animals used; the second the No. of determinations; the third the wt of tumor expressed as % of body wt.

† All values are means ± standard errors S.E. $= \sqrt{\frac{\Sigma(x)^2}{n(n-1)}}$.

TABLE II. Nucleic Acid Changes in Tissues of Swiss Mice with Transplantable Sarcoma.

Tissue	Controls 7:1 *	1st wk 40:1 : .6 *	2nd wk 4 : 1 : 3.4 *	3rd wk (40:1.*
		RNA, mg/g dry wt		
Sarcoma		52.5	44.7	31.2
Liver	33.5 ± .9†	40.2	38.4	40.2
Kidney	24.6 ± .9	21.5	22.1	24.3
Lung	8.6 ± 1.5	16.9	14.7	21.9
Spleen	40.7 ± 1.6	39.6	36	36.2
		DNA, mg/g dry wt		
Sarcoma		43.5	38.3	36.2
Liver	9.8 ± .8†	12.3	11.8	17
Kidney	21.9 ± 1.9	21.5	25.6	33.7
Lung	17.5 ± 2.2	26.5	33.9	37
Spleen	141.1 ± 18.5	103	89	90.5

* The first No. in parenthesis indicates No. of animals used; the second the No. of determinations; the third the wt of the tumor as % of body wt.

† All values are means ± standard errors.

procedure used in our experiments is a modification of the methods of Hotchkiss(11), and of Vischer and Chargaff(12), it will be described in some detail. After removal of phospholipids and of acid-soluble phosphorus the tissue (60-100 mg dry wt) was treated with 70% perchloric acid(13). Chromatograms were developed on strips of Whatman No. 1 filter paper, using two different solvent systems. In one case, strips were developed with a mixture of 14% water in n-butanol (v v)(11). This system gave a clean separation of guanine (Rf 0.12), cytosine (Rf 0.22), and thymine (Rf 0.48), but no clear separation of uracil and adenine. The second system consisted of a mixture of 7 volumes of n-butanol and 1 volume of 1 N HCl. This effected a separation of uracil (Rf 0.32) and thymine (Rf 0.45). The position of the substances was determined by observation of the chromatogram under ultraviolet light(13). Cytosine and uracil were eluted with 5 ml of distilled water and determined colorimetrically(14). Thymine was eluted with 0.1 N HCl and then estimated spectrophotometrically(12).

Results. The results of the experiment with sarcoma 180 in the 2 strains of mice are summarized in Tables I and II. They show that in the dba animals, (Table I) there are no changes in the nucleic acid content of any of the organs studied at the end of the first week. Two weeks after transplantation, there is a pronounced rise in the RNA and DNA of the liver and the lung, whereas in the kidney, only the DNA shows this increase. On the other

TABLE III. Nucleic Acid Changes in Tissues of Mice (dba) Bearing Melanoma S-91

Group	No. wk after inoc.	No. of animals	Tumor wt as % of body wt	Liver RNA mg/g dry wt	Liver DNA mg/g dry wt	Kidney RNA mg/g dry wt	Kidney DNA mg/g dry wt	Lung RNA mg/g dry wt	Lung DNA mg/g dry wt	Melanoma RNA mg/g dry wt	Melanoma DNA mg/g dry wt
I	4	5	.65	29.1	12.6	27.2	24.4	11.7	28.7	56.8	55.1
	5	5	.79	29.8	11.3	24.8	24.6	11.4	27.4	49.2	57.2
	6	5	4.78	38.6	14.9	27	35	11.1	29.4	50.5	64.1
	7	4	10.15	34.2	17.1	23.6	38.4	16.4	30.8	43	66
	8	4	13.10	37.4	17.4	23.8	32.8	17	37.4	42.7	61.5
	9	4	11	43.6	17.7	22.4	27.4	18.3	54	23.2	44
II	4	5	.58	30.2	10.2	26.2	18.8	10.5	27.3	56	48
	5	5	.67	32.6	11	24.8	20	8	21.2	57.2	44.5
	6	5	1.80	30.6	9.6	25.8	20	11.1	25.8	54.3	44.2
	7	5	2.26	35.7	8.4	27.7	22.5	13.1	29	49.7	34.4
	10	4	9.26	37.6	12	24.2	36.2	12.8	38.5	50.2	54.9
	12	4	9.95	35.6	12	22.4	33.8	15	32.8	43	54.3

hand, in the Swiss strain (Table II) the changes in the liver and lung are significant after the first week. If the maximum increases are compared, we find that in the dba mice, the content of RNA in the liver has increased by 72%, and of DNA by 115% over the controls. The corresponding increases in the liver of the Swiss mice are 20% and 73%. On the other hand, the increases in the lung nucleic acids in the Swiss mice are considerably higher than in the dba mice. Apparently, we are dealing here with a strain difference.

The experiments with the melanoma were performed on 3 different occasions. In the initial experiment, approximately 10-week-old animals were used. The tumor was palpable after 3 weeks, but thereafter its growth was slight and irregular. As there were no changes in the nucleic acids at the end of 8 weeks, the experiment was discontinued. The experiment was repeated twice. A 6-week-old tumor was transplanted into 5- to 6-week-old animals. In spite of the fact that the conditions under which the experiments were carried out were the same, the growth of the tumor and the changes in the tissue nucleic acids were different in the 2 experiments. Therefore, the data are given separately in Table III. In one experiment (Table III, Grp. I) significant changes were first observed in the liver at the end of 6 weeks, when the tumor size was 4.8% of the body weight. Thereafter, a progressive increase in the RNA and DNA of the liver and lung was observed. The kidney DNA behaved somewhat differently. There was a sudden increase of 61% at the end of 6 weeks; of 77% at the end of 7 weeks, and then a gradual decrease. In this group, the animals survived for approximately 10 weeks after the transplantation of the tumor. In the other experiment (Table III, Grp. II) the growth of the tumor was slower, and the animals survived for 14 weeks. Furthermore, the increase in nucleic acid content was not of the same magnitude. It follows from these data that the nucleic acid changes are related to the size of the tumor and to its rate of growth.

Similar changes in the nucleic acid content were observed in the tissues of animals bearing spontaneous mammary tumors(18). The data obtained in 2 groups of 5 females of the Swiss strain which were 10 months old and 2 groups of 5 litter mate controls without tumors are shown in Table IV. They show that the liver nucleic acid content is lower than that found in the controls in the other experiments. This may be due to the fact that these animals were older. In the experimental animals, of the tissues studied, the lung showed a significant rise in its nucleic acid content. If the ratio RNA DNA in the tumor is calculated from the values given in Tables I, II and III, it will be found that it generally falls as the tumor growth progresses. Table V shows the nucleic acid, purine, and pyrimidine content of melanoma S-91. It may be seen from these data that both RNA and DNA values are lower in the necrotic portion as compared to the non-necrotic portion of the same tumor. The values for purines and

TABLE IV. Nucleic Acid Changes in the Tissues of Swiss Mice Bearing Spontaneous Mammary Tumors in mg/g Dry Wt.

Tissue	Controls		Experimental	
	RNA	DNA	RNA	DNA
Liver	18.8; 22.2	5.3; 6.4	20 ; 21.2	8.8; 9.1
Kidney	17.8; 18	12 ; 13.7	18.6; 23.4	18.6; 21.8
Lung	12.4; 12	15.9; 16.9	15.7; 16.7	32 ; 30.6
Spleen	31.2; 29.7	95.6; 116.7	44.7; 39.2	98.8; 100.4

TABLE V. Nucleic Acid, Purine and Pyrimidine Content of Melanoma S91.

Age of tumor, wk	No. of tumors studied	RNA	DNA	Guanine	Adenine	Uracil	Thymine	Cytosine
		mg/g dry wt				μg/100 mg dry wt		
4	5	56	48	13.2	12.3	—	—	—
5	5	57.2	44.5	12.35	9.5	—	—	—
6	5	54.3	44.2	11.69	9.09	245	588	1052
7	4	49.7	34.4	12.02	9.63	243	433	946
10	4	50.2	54.9	13.12	9.65	178	573	953
*		55.4	61.6	13.50	10.46	245	626	1137
†		43.3	52.3	11.78	7.92	159	439	851
12	4	43	52.3	10.10	8	193	533	935
*		51.8	61.7	10.45	8.20	257	599	1018
†		39.2	45.2	8.78	7.26	155	482	793

* Non-necrotic portion. † Necrotic portion.

pyrimidines also indicate a similar trend. The drop in the ratio RNA/DNA may therefore be attributed to a decreased concentration of RNA in both the necrotic and the non-necrotic portions of the tumor, while DNA content decreases only in the necrotic portion.

Discussion. Reddy and Cerecedo(4) observed an increase in the nucleic acid content of the liver, lung, and kidney in mice during gestation. In the present paper it is shown that changes in the nucleic acids are also induced by neoplastic growth. These changes, however, are more pronounced than those found during gestation. Furthermore, the extent of these changes appears to be related to the rate of growth of the tumor. The observations of Greenstein and Andervont (15) in connection with liver catalase are of particular interest in this regard. They found that the decrease in liver catalase activity in mice was progressive with the growth of the tumor, and that the drop was most pronounced in animals bearing the most rapidly growing tumors. It is worthy of note that, in contrast to the effect of pregnancy on the nucleic acid content of various tissues observed in the mouse, embryonic tissue has no effect on the catalase activity of the liver(16). The work of Daoust and Cantero(17) is also in harmony with our findings. They found an increased activity of ribonucleopolymerase in the liver of the rat during the precancerous stage, and this increase was related to the RNA content of this organ.

Summary. 1. The nucleic acid content of various tissues has been determined in mice of the Swiss and dba strains bearing transplanted sarcoma 180 (Crocker), in dba mice bearing malignant melanoma S-91, and in Swiss mice bearing spontaneous mammary tumors. 2. In the mice with transplanted tumors, an increase in the pentose nucleic acid (RNA) and desoxypentose nucleic acid (DNA) content of the liver and lung was observed, whereas the kidneys showed a similar rise in the DNA. In the mice with mammary tumors, the most marked increase was found in the lung nucleic acids. 3. The changes of the nucleic acids in the dba mice could be related to the size and the rate of growth of the tumor. 4. The ratio RNA/DNA in the tumor usually dropped with the growth of the tumor. This drop is attributed to a decreased concentration of RNA in both the necrotic and non-necrotic portions of the tumor, whereas the DNA content dropped only in the necrotic portion. 5. The nucleic

acids, purines, and pyrimidines in malignant melanoma S-91 have been determined at various stages of its development. The lower values for uracil and thymine found in the necrotic portion of the tumor as compared with those found in the non-necrotic portion point also to a drop of both RNA and DNA in the necrotic portion.

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ACTH and Cortisone Aggravation or Suppression of the Febrile Response of Rabbits to Bacterial Endotoxin. (19182)

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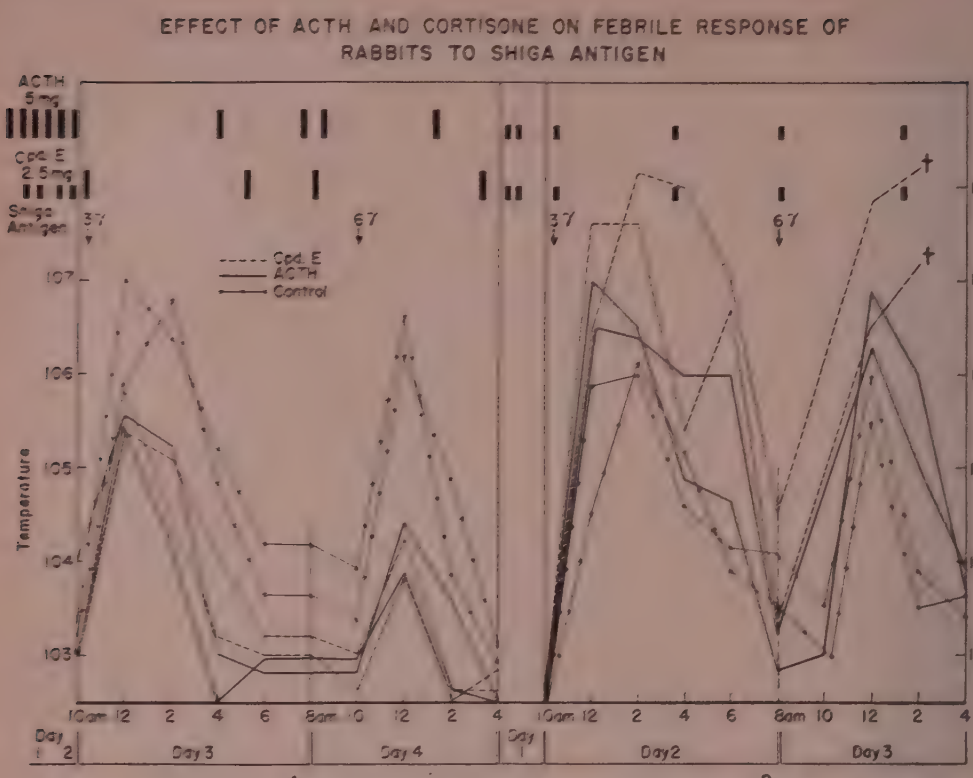
Recent reports(1,2) indicate that ACTH and cortisone reduce the febrile response of rabbits to typhoid vaccine and, furthermore, cortisone has been shown to markedly reduce the temperature of patients with typhoid fever(3). Since the somatic antigens of Salmonella and Shigella organisms account for practically all of the toxicity of these bacteria, and may be responsible for the febrile reactions accompanying infections produced by these organisms(4-6), it was decided to investigate the effects of ACTH and cortisone on the response of rabbits to intravenous injections of a bacterial endotoxin, the purified somatic antigen of *Shigella dysenteriae* (*Shiga*)(7). Since some recent experiments suggested that ACTH and cortisone had no effect on the febrile response of rabbits to crude endotoxin prepared from *S. typhosa*(8) in contrast to the results reported by the other investigators(1,2), special attention was paid in these studies to an analysis of the time-dose relationship of the bacterial endotoxin and the ACTH or cortisone since the variations in effect produced could be due to differences in dosage and time of injection of the hormones.

Method. Young adult, male, albino rabbits weighing from 2.5 to 3 kg were given preparatory treatment with ACTH and cortisone* in groups of 2 animals each. One

* The ACTH was supplied through the kindness of Armour and Co. and the cortisone by Merck and Co.



Fever reactions, following the first injection of pyrogen in Experiment A, are listed in terms of fever units.

	Fever units	
	Exp. A	Exp. B
Control	15.8	12
Cortisone	4.1	22.3
ACTH	3.4	31.6

group (A) received 15 mg of ACTH in 3 doses or 5 mg of cortisone in 2 doses for 2 days prior to injection of antigen. On the 2 days of the injection of antigen, the rabbits received twice daily injections of 5 mg of ACTH or cortisone. Another group (B) received 5 mg of ACTH or 5 mg of cortisone in 2 divided doses on the day before treatment with antigen, and for the 2 days of the experiment. The ACTH was administered intramuscularly and the compound E subcutaneously. The endotoxin was prepared from *Sh. dysenteriae (Shiga)* by the method of phenol extraction as previously described(7), and was given intravenously in doses of 3 or 6 μg. Hourly rectal temperatures were taken until the temperatures returned to normal levels.

Results. The animals who received the larger doses of ACTH and cortisone (Exp. A) for at least 2 days prior to the injection of antigen showed a depression of the febrile response compared to that of the control animals. The inhibition was observed on the first and second days during which ACTH and cortisone were continued. In contrast to this was the aggravation of fever in the animals (Exp. B) treated with less intensive doses of ACTH and cortisone for 24 hours prior to the injection of toxin. The augmentation of fever was noted on both days of the endotoxin test injections. The cortisone-treated animals showed a more marked effect and these 2 rab-

bits died in hyperthermia following the second injection of the test antigen. The contrast in the effects obtained in these 2 experiments is noted in the chart of the febrile response in terms of fever units† on the first day of the 2 experiments for one animal of each pair treated.

The febrile reactions of control animals in Exp. B were somewhat less marked than those in Exp. A. This difference is probably due to variations in the room temperature which affect the body temperatures of the rabbits as indicated by the lower initial temperatures of all rabbits in Exp. B. Environmental temperature, however, affects experimental as well as control animals involved in the same experiment so that the influence of hormone treatment is not altered. These observations were confirmed in several similar experiments.

Discussion. The previously noted(1,2) antipyretic action of ACTH and cortisone for the endotoxins of enteric bacilli has been confirmed. In contrast to these previous reports of protective action, an aggravation of the febrile response is noted in the ACTH and cortisone-treated animals in whom the administration of these compounds was less intensive. It appears likely that if the proper time-dosage relationships had been chosen no significant effects might have been observed on the febrile response and the results would have corroborated the findings of Jackson and Smadel(8) with a crude typhoid endotoxin. The timing and quantity of ACTH or cortisone appears then to be an all-important factor in the determination of the effects of these materials on the febrile response of rabbits to these endotoxins. This appears to account for the discrepancies in the results previously reported.

In the light of the previous experiments demonstrating that repeated injections of bacterial endotoxins result in the development of a tolerance to their toxic effects which is not the result of antibody formation(4,5,7), the finding that ACTH or cortisone, in proper dosage, results in an increased resistance to the pyrogenic effects of endotoxins may indicate that a change in the functional state of the adrenal gland may be responsible, at least in part, for this tolerance.

Summary. (1) ACTH in divided doses of 15 mg per day and cortisone (5 mg per day) for 48 hours prior to the intravenous injection of a bacterial endotoxin (*Shiga dysenteriae*) and injection of 10 mg of either compound during the 2-day test period resulted in a significant lessening of the febrile response in the treated as compared to the control animals. (2) Rabbits given smaller doses of ACTH (5 mg per day) or cortisone (5 mg per day) for 24 hours prior to the injection of antigen, and continued through the test period, demonstrated an aggravated febrile and systemic response to the injected endotoxin as compared to the controls. (3) The significance of this time-dose relationship in the interpretation of experiments involving the use of ACTH and cortisone and bacterial endotoxins is discussed.

† A fever unit is a degree-hour of temperature rise over 104°F.

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Rickettsiostasis in Fertile Eggs from Use of Antibiotic Residues in Poultry Feeds.* (19183)

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During the past 8 months we have obtained irregular and usually very scanty growth of murine typhus rickettsiae in the yolk sacs of fertile eggs, in striking contrast to the uniformly heavy infection previously observed over a period of 7 years. This definitely altered susceptibility coincided with the adoption of new types of feeds by the flock owners from whom we have obtained our eggs. The feeds used in recent months are supplemented by fermented mashes left over from the manufacture of antibiotics and vit. B_{12}.

The experiments to be reported here were planned to test the possibility that our recent failure to cultivate rickettsiae satisfactorily in eggs might be the result of the factors newly introduced into the diet of laying hens. Another objective was to determine the effect of these new factors on the multiplication of Influenza A virus.

Materials and methods. To carry out this investigation it was necessary to obtain fertile eggs from a flock that had never been fed the new type of ration. After visiting many small farms in the vicinity of our laboratory, a flock (Flock A) meeting these requirements was located. The owner of this flock had supplemented his ground corn only with meat meal, fish meal, dried milk and minerals, following the procedures used in past years in the preparation of poultry feeds. It was possible to trace this flock back to the hatchery, where records showed that Flock A was only a small portion of the original hatch, the remainder (Flock B) having been sold to a nearby poultry man. Flock B had received, since hatching, a diet identical with that given to Flock A, but supplemented with 1 pound of antibiotic residue per 100 lb of final ration. This antibiotic residue was guaranteed by the processor to contain, by assay, not less than 1 g of antibiotics (aureomycin and terramycin) per lb, as well as an unspecified amount of vit B_{12}. Aureomycin and terramycin are known to be powerful rickettsiostatic agents. Eggs from Flock A were gathered at 12 hr intervals and stored at approximately 15°C, at which temperature they remain viable for 4 to 6 weeks. After gathering eggs for several days, (Group 1) the diet of the hens of Flock A was changed to the new type described above, which Flock B had received since hatching. Six days later, eggs were again gathered twice daily for 2 days (Group 2). Flock A was continued on the new diet and eggs were gathered and stored at the end of the second week (Group 3) and at the end of the third week (Group 4). Eggs from Flock B were collected and stored during the same periods (Groups 1, 2, 3, and 4). After the last eggs had been gathered all, with the exception of those used for influenza studies, were incubated for 5 days at 37.5°C and then injected with rickettsiae. The methods used for preparing the inoculum, infecting fertile eggs, making and staining yolk sac smears, and determining the degree of infection were those described in previous papers(1,2). Five eggs from Group 1, Flock A, and 5 from Group 1, Flock B, were incubated for 11 days at 37.5°C and then tested for ability to support the growth of the PR8 strain of influenza virus. The technique used for inoculation and measuring growth was that recently published by Blumenthal *et al.*(3).

Results. The effect of changing the diet of Flock A hens from the original one, presumably free from antibiotics, to the new one containing aureomycin and terramycin, is shown in Table I. The eggs in Group 1, fed the original diet, showed the characteristic pattern of heavy infection, with embryonic death on or before the 7th day after injection. The eggs collected after feeding the new diet

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TABLE I. Growth of Rickettsiae in Embryonated Eggs from Hens Fed a Diet [illegible] and Without Antibiotics.

Age of embryos	No. and Position Group 1	Group 2	Group 3	Group 4
		1 wk (Group 2)	2 wk (Group 3)	3 wk (Group 4)
5		Rickettsiae inoculated		
9	1, 2, 3, 3	1, 2, 2	†, †	0, 0
10	3, 3, 4, 4, 4, 5	2, 2, 3, 3, 4, 4	0, 0, 0, †	0, 0, †, †
11	3, 4, 4, 4, 4, 4, 4, 4, 4, 5, 5, 5, 5, 5, 5, 5	2, 3, 3, 3, 3, 3, 4, 4, 4, 4, 4, 4, 5, 5, 5, 5	†, †, 1, 1, 1, 2, 3, 3	0, 0, †, †
12	4, 4, 4, 5, 5, 5, 5, 5, 5, 5, 5, 5, 5, 5	2, 3, 4, 4, 4, 4, 4, 5, 5, 5	1,* 1,* 2,* 2,* 3*	
13		4, 4, 5, 5, 5, 5	2,* 3,* 3, 4,* 4	0,* 0,* 0,* 0,* †*
15			3, 3,* 3,* 4,* 4	0,* †*, †*, †*, 2**
17			2,* 3,* 4,* 4,* 4	0,* †*, 1,* 1,* 2*
18			†*, 2,* 3,* 4,* 4, 5	0,* 0,* 0,* 0,* 0,* 0,* 0,* 0,* 0,* 0,* †*, †*, 1,* 1,* 2,* 3*

Each figure represents an individual egg.
* Embryos alive at time of examination.
0 = No rickettsiae recognized.
† Less than one rickettsiae per oil immersion field.
1, 1-10 rickettsiae per oil immersion field; 2, 10-100; 3, 100-1000; 4, 1000-5000; 5, 5000-10000.

for 6 to 7 days showed little or no alteration in susceptibility, but those collected toward the end of the second week showed marked resistance to rickettsial growth, while those collected near the end of the third week (Group 4) showed almost complete resistance. All 4 groups of eggs from Flock B showed resistance comparable to that seen in Group 4 eggs of Flock A. The 24 hr infectivity titres of pooled chorio-allantoic fluids from eggs of Group 1, Flock A and of Group 1, Flock B, inoculated with Influenza A virus, were $10^{-9.2}$ and $10^{-9.5}$ respectively (differences not considered statistically significant).

Discussion. We did not assay the dietary supplements used in these experiments for antibiotics content. If we accept the assay figures given on the package label by the manufacturer, 1 g of antibiotic would be present per 100 lb of final ration, and if we assume that these antibiotics (aureomycin and terramycin) are incorporated in the yolk at rates comparable to those established for dyes(4), labeled (C^{14}) sodium acetate (5), and vitamins(6), it is clear that rickettsiostatic concentrations of these antibiotics should be present in the yolk.

Most present day poultry feeds differ from those used in past years in that they contain antibiotics. It is unlikely that the vit. B_{12} content has changed greatly, since the older rations were rich in this compound. We cannot, however, entirely exclude the possibility that this vitamin, or some other unknown agent may have been responsible for the rickettsiostatic effect noted.

In other experiments not reported here we have found only slight or moderate inhibition of rickettsial growth in eggs from flocks receiving diets of the new type. In these diets, however, the processor merely stated that unspecified antibiotics were present in unspecified amounts; no assay figures were given. There is great variation in the composition of currently used poultry feeds. Moreover, some breeders intentionally withhold antibiotics from hens during the laying period (using supplements containing only B_{12}) while others continue to use diets containing antibiotics. For this reason irregular results may be expected in the cultivation in eggs of rickettsiae, and perhaps also of other infective agents and tumors, unless a standardized diet is used.

Conclusions. The introduction of antibiotic mash containing aureomycin and terramycin

into the diet of a flock of laying hens resulted in progressively increasing resistance of the eggs to rickettsial growth, the resistance becoming almost complete at the end of 3 weeks. Similar dietary factors had no appreciable effect on the growth of Influenza A virus in eggs. These results, with other data submitted, indicate that fertile eggs obtained from commercial hatcheries may, under present conditions, be unsatisfactory for controlled microbiological studies.

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Counteraction of Cortisone Inhibition of Body, Hair and Thymus Growth by Vitamin B_{12} and Aureomycin.* (19184)

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Large doses of cortisone have been shown to inhibit both body(1,2) and hair growth (3,4) in rats. These effects can be at least partially attributed to the reduction in availability of protein to the organism(5,6). Large doses of cortisone have also been reported to reduce normal food intake in rats(7), which may further contribute to reduced body and hair growth. Vit. B_{12} partially or completely counteracted growth-inhibition in rats induced by administering large doses of thyroid-active substances(8-10), diethylstilbestrol (11), or 0.1% thiouracil(12). Several antibiotics have also been shown to be effective in prolonging survival time(13) and overcoming growth-inhibition(14) in hyperthyroid rats. In general, these studies are believed to indicate that marked changes in estrogen or thyroid levels within the body can increase the need for vit. B_{12} and perhaps other factors by the organism.

It was the purpose of the present study to determine whether vit. B_{12} and aureomycin could prevent large doses of cortisone from depressing body and hair growth in rats fed a ration deficient in vit. B_{12}. If large amounts of cortisone can aggravate an existent dietary deficiency, and thus account to some extent for inhibition of body and hair growth, then supplementing the diet with the missing nutrients should help to prevent these manifestations.

Methods. A total of 130 immature male albino rats of the Carworth strain were used in 3 separate experimental trials. All rats were divided into uniform groups by weight of 10 each, and were placed for 30 days on the following vit. B_{12}-deficient ration: yellow corn meal, 35%; ground wheat, 25%; linseed oil meal, 10%; soybean oil meal, 20%;‡ alfalfa leaf meal, 6%; brewers' yeast, 3%; and table salt, 1%. *Cortisone acetate* (*Cortone*)§ was injected subcutaneously, daily, in a 0.1 ml volume of saline containing 0.5 mg of the hormone. Crystalline vit. B_{12}§ was

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† With the technical assistance of Mrs. R. C. Ogle.

‡ Low fiber, solvent extracted, containing 50% protein. Manufactured by Archer-Daniels-Midland Co., Minneapolis, Minn.

§ Cortone and crystalline vit. B_{12} were furnished through the courtesy of Dr. D. F. Green, formerly of Merck and Co., Rahway, N. J.

TABLE I. Effects of Vitamin B_{12} and Aureomycin on Cortisone Action in Male Rats.

Group	Treatment	Avg body wt Orig., g	Avg body wt Final, g	Avg food intake Total, g	Avg food intake Per g gain body wt, g	Avg adrenal wt Actual, mg	Avg adrenal wt Per 100 g body wt, mg	Avg thymus wt Actual, mg	Avg thymus wt Per 100 g body wt, mg
1	Controls	54.5 (30)*	141.1 (28)*	293.1	3.4	22.3	16 ± .9†	277.8	198.5 ± 10.7†
2	200 μg B_{12}	57.8 (10)	158 (9)	299.7	2.9	25.7	16.3 ± 1	328.1	207.7 ± 13.6
3	.005% aureo.	58.2 (10)	171.8 (9)	315.3	2.7	28.5	15.6 ± .8	369.3	215 ± 10.7
4	200 μg B_{12} + .005% aureo.	57.9 (10)	178.3 (9)	351	2.9	27.9	15.6 ± .7	365.6	205 ± 9.6
5	.5 mg Cortone	54.8 (30)	121.3 (25)	260.4	3.9	13.2	10.2 ± .8	142.4	110.5 ± 8.3
6	.5 mg Cortone + 200 μg B_{12}	54.6 (30)	143.1 (27)	313.4	3.5	15	10.6 ± .4	223.1	160 ± 10.6
7	.5 mg Cortone + .005% aureo.	53.4 (20)	132.7 (19)	297.6	3.6	14.9	11.3 ± .8	195	144.8 ± 13.1
8	.5 mg Cortone + 200 μg B_{12} + .005% aureo.	53.4 (20)	151.8 (20)	325.4	3.3	17.3	11.6 ± .5	237	157.4 ± 11.8

* No. of rats per group. † Standard error of mean.

mixed into the ration in amounts of 200 μg per kilo, and aureomycin HCl¶ in amounts of 0.005%. Body weight and food consumption were measured on alternate days. At the end of the 30-day experimental period, the rats were killed and the adrenals, spleen, thymus and testes were removed and weighed. All rats were housed in an air-conditioned animal room at a temperature of 75 ± 1°F. The effects of cortisone and the dietary supplements on hair growth were determined in only 2 experiments. The hair was uniformly removed from the backs of the rats, from neck to tail, with a razor. In one experiment, the hair was not removed until the 18th day, leaving 12 days for regrowth of hair. In the other experiment, the hair was removed on the first day, leaving 30 days for regrowth of hair. At the end of the experiments, each group was laid side by side and each rat was rated individually for degree of hair regrowth. The ratings for each group were averaged. The following rating scale was used: 0, no regrowth; 1, slight regrowth; 2, moderate regrowth; 3, marked regrowth; 4, complete regrowth.

Results: The data from the 3 experiments were essentially similar and have been combined in Table I. It can be seen that when vit. B_{12} (Group 2) or aureomycin (Group 3) were fed individually or together (Group 4), they increased body weight gains above the controls (Group 1). The vitamin and antibiotic increased food intake more than is apparent here, since the controls of the individual experiment consumed only an average of 271 g of food per rat. Vit. B_{12} and aureomycin reduced the amount of food required for each g of gain in body weight. Adrenal and thymus weights were not affected by these dietary supplements.

Cortisone (Group 5) reduced body growth and food intake, and decreased the efficiency of transforming food into body weight gains. Adrenal and thymus weights were significantly reduced, the latter weighing only about half as much as in the controls. Vit. B_{12} (Group 6) completely and aureomycin (Group 7) partially counteracted the cortisone-induced inhibition of body growth and food intake, and both increased growth efficiency. The combination of the vitamin and antibiotic (Group 8) was more effective in all these respects than either substance alone. Although neither substance prevented the decrease in adrenal weight, they were both partially but significantly effective in counteracting the cortisone-induced decrease in thymus weight. This was found to be true in all 3 individual experi-

¶ Crystalline aureomycin HCl was kindly supplied by Dr. T. H. Jukes of Lederle Labs. Division, Pearl River, N. Y.

ments. The data pertaining to spleen and testes have not been presented in tabular form since neither cortisone (in the dose given) nor the dietary supplements significantly altered the weights of these organs.

The average rating for regrowth of hair in the rats from whom hair had been removed 12 days previously was as follows for each group: controls, 3.0; .5 mg cortisone, 1.1; .5 mg cortisone and 200 μg vit. B_{12}, 2.1; .5 mg cortisone and .005% aureomycin, 2.2; .5 mg cortisone, 200 μg B_{12} and .005% aureomycin, 2.7. The ratings for the rats from whom hair had been removed 30 days previously are given in the legend under Fig. 1. In general, the results of the two experiments are in agreement.

Discussion. In 3 similar experiments it was found that a relatively large dose of cortisone injected into immature rats on a ration deficient in vit. B_{12} aggravated the existent dietary imbalance. This was indicated by the partial retardation of body growth and appetite and in reduced ability to transform food into body weight gains. The incorporation of vit. B_{12} into the ration completely counteracted the cortisone-induced inhibition of body growth, although growth did not reach the level of the non-injected rats given vit. B_{12}. When vit. B_{12} and aureomycin were given together to the cortisone-injected rats, growth proceeded beyond the level of the non-injected control rats.

FIG. 1. From top to bottom, average rating for regrowth of hair for each group was as follows: controls, 4; .5 mg cortisone, 1.7; .5 mg cortisone and 200 μg B_{12}, 2.7; .5 mg cortisone and .005% aureomycin, 2.3; .5 mg cortisone, 200 μg B_{12} and .005% aureomycin, 3.2.

The ability of vit. B_{12} and aureomycin to partially counteract suppression of hair growth in these experiments was associated with their ability to simultaneously overcome inhibition of body growth. Hair and body growth were not suppressed to the same degree by cortisone, nor were these inhibitory effects counteracted to the same extent by vit. B_{12} and aureomycin. However, it was notable that on an individual as well as on a group basis, the heavier rats invariably showed more regrowth of hair than the lighter rats, and vice versa. The parallelism of these actions by cortisone and the two dietary supplements on hair and body growth suggests that similar mechanisms were in operation.

It is of considerable interest that vit. B_{12} and aureomycin partially protected the thymus against atrophy by cortisone. We have previously observed (unpublished data) that the decrease in spleen weighed induced by injecting 1 mg daily of cortisone into young rats can also be partially prevented by feeding extra quantities of vit. B_{12}. Pentz *et al.*(15) reported that liver and vit. B_{12} partially counteracted the loss in thymus weight of thyroid-fed rats having greatly hypertrophied adrenals. Similarly, several antibiotics were shown to partially or completely prevent loss of thymus weight in hyperthyroid rats(16). Inasmuch as the adrenal atrophy induced by cortisone in these experiments was not at all prevented by vit. B_{12} or aureomycin, it appears that the protective action on the thymus gland was a direct one. It would be interesting to determine whether vit. B_{12} or anti-

biotics would protect other lymphoid tissues against cortisone action.

Experiments now underway in this laboratory indicate that large doses of cortisone (1 mg daily) may induce deficiencies of vit. B_{12} even when a diet normally adequate in this factor is fed. Extra amounts of vit. B_{12} in an otherwise adequate diet have also enabled rats to partially overcome inhibition of growth and appetite induced by feeding large doses of diethylstilbestrol or thyroprotein(11). In general, these results are believed to indicate that marked changes in estrogen, thyroid or cortisone levels within the body may alter the nutritional requirements of the organism.

The mechanisms by which vit. B_{12} and aureomycin protected the rats against cortisone in these experiments remain to be elucidated. It is pertinent, however, that vit. B_{12} has been shown to favorably influence protein synthesis(17,18) and to increase nitrogen retention(19), while aureomycin may increase the availability of vit. B_{12}(20,21) and niacin(22) to the organism.

Summary. (1) Cortisone was injected in daily doses of .5 mg for 30 days into 130 immature male albino rats on a vitamin B_{12}-deficient diet. The depressions of body, hair, and thymus growth which resulted were completely or partially prevented by incorporating 200 μg of vit. B_{12} pr kilo of ration or .005% aureomycin. (2) Vit. B_{12} was more effective in these respects than aureomycin, and the combination of the two substances was more effective than either alone. (3) The favorable actions of the vitamin and antibiotic were accompanied by an increase in food consumption and greater efficiency in converting food into body weight gains. (4) It is concluded that when large doses of cortisone are injected into rats on a vit. B_{12}-deficient diet, the deficiency becomes aggravated and thus accounts at least in part for the observed inhibitions of body and hair growth. The mechanisms by which vit. B_{12} and aureomycin protect the thymus against cortisone action are as yet unknown.

Addendum. While this paper was in press, Wahlstrom and Johnson (PROC. SOC. EXP. BIOL. AND MED., 1951, v78, 112) independently reported that injections of relatively large doses of cortisone into baby pigs fed a vit. B_{12}-deficient diet, aggravated the deficiency. Body growth and survival rate were reduced and excretion of vit. B_{12} into the urine was increased. This is believed to confirm the opinion that large doses of cortisone increase requirements for vit. B_{12} and possibly other factors.

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Isolation of a Psittacosis-Like Agent from the Blood of Snowy Egrets.* (19185)

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(Introduced by Morris Schaeffer.)

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During the course of an investigation to determine the animal reservoirs of eastern equine encephalomyelitis, many wild birds inhabiting the swamps and coastal areas of Louisiana were bled, and serum from each was inoculated intracerebrally into six 3-week-old Swiss mice(1). On June 27, 1950, blood was obtained from 24 nestling snowy egrets (*Egretta candidissima candidissima* (Gmelin)) and from several other species of wading birds on an island in Barataria Bay off the Gulf coast of eastern Louisiana. Several mice inoculated with sera from 2 snowy egrets developed ruffled fur and irritability followed by weakness, convulsions, and paralysis, finally resulting in death. No bacterial growth was observed when brains from these mice, and from subsequent passages, were cultured on blood agar plates, in serum or dextrose broths.

Further intracerebral passage in mice established the presence of an infectious agent, or agents, producing death within 2-7 days after inoculation. A great reduction in titer of the agent was observed on filtration through a Seitz EK pad, indicating that all but a few of the infectious particles were retained by this type filter. No evidence of *in vitro* neutralization was obtained when this agent was tested against antisera to eastern equine encephalomyelitis, western equine encephalomyelitis, Venezuelan equine encephalomyelitis, St. Louis encephalitis, lymphocytic choriomeningitis, or encephalomyocarditis viruses.

Sections made from these brains revealed a heavy infiltration of mononuclear cells into the leptomeninges. Many of these cells contained clusters of minute coccoid bodies resembling the elementary bodies of the psittacosis-LGV group of organisms. Macchiavello-stained smears of livers and spleens of intraperitoneally inoculated mice confirmed this impression. Intranasal inoculation into mice produced death, and autopsy revealed widespread pulmonary involvement and the presence of many bodies morphologically and tinctorially like those of psittacosis.

Limited host range studies carried out with material derived from both isolations revealed identical pictures. Material from both isolations was highly fatal for mice by the intracerebral, intraperitoneal, intramuscular, and subcutaneous routes. Guinea pigs and hamsters tested only by intracerebral and intraperitoneal routes of inoculation also succumbed. Inoculation into the yolk sac of 6-day chick embryos was followed by death in 3-5 days. Smears made from the yolk sacs revealed large numbers of elementary bodies. Inoculation into the allantoic cavity of 10-day-old chick embryos resulted in death in 3-7 days. Rabbits inoculated intracerebrally and intraperitoneally exhibited no obvious signs of infection. Temperatures were not taken, however, nor was the serological response studied. Four chickens inoculated intraperitoneally with a 1:10 dilution of freshly harvested yolk sac material showed toxic signs, and 3 died within a few days.

Yolk sac antigens made by the method of Nigg *et al.*(2) fixed complement in the presence of psittacosis antiserum. A rise in complement fixing antibodies against psittacosis and LGV antigens was observed in immunized guinea pigs.

When mice infected by the intraperitoneal route were treated with penicillin, there resulted a significant reduction in mortality rate as compared with untreated controls.

Guinea pigs immunized with material from one of the isolations failed to show evidence of infection when challenged with the other. This fact, combined with the fact that both

* This note constitutes part of an investigation on encephalitis in Louisiana conducted by the Virus and Rickettsia Section, Montgomery, Ala.

agents were isolated from the same species on the same day and with the observation that both agents possessed similar host ranges and histopathological reactions, indicated close resemblance, if not identity, of the two.

The high fatality rate following intraperitoneal inoculation in guinea pigs and intramuscular and subcutaneous inoculation in mice, and the toxicity for chickens inoculated intraperitoneally(3) suggested a relationship to the virus of Louisiana pneumonitis isolated by Olson *et al.*(4-9) during an epidemic of severe pneumonitis among residents of the bayou region of Louisiana in 1943.†

To check on such possible relationship, each of 4 guinea pigs immunized to the newly isolated agent were inoculated intraperitoneally with 1 ml of a 10^{-1} dilution of Louisiana pneumonitis virus in mouse brain. No evidence of infection was observed. The temperature of all 4 control guinea pigs rose above 105° F, and 2 died. The 2 survivors from this control group were kept until they returned to apparently healthy condition and were given an intraperitoneal inoculation of the new agent. They showed no evidence of infection, while 2 control guinea pigs died. The infectivity of material from the two isolations was compared with Louisiana pneumonitis virus and the 6 BC strain‡ of psittacosis by inoculation through various routes into mice, and by intraperitoneal inoculation into guinea pigs. The pattern of infection produced by the new agent was remarkably similar to that of the Louisiana pneumonitis (Borg) virus and was distinct from that produced by the 6 BC strain. The latter was not as highly virulent for guinea pigs by intraperitoneal inoculation, and proved innocuous for mice by the intramuscular and subcutaneous routes.

Although no work had been done in the laboratory with any of the psittacosis viruses for over a year and a half, it was, nevertheless, deemed necessary to rule out the possibility of accidental contamination. The original serum specimens were reinoculated into mice, and the virus again was recovered from one of the specimens. The other specimen produced symptoms in 3 of 6 mice inoculated. One was killed, but its brain was accidentally discarded. The other 2 recovered and no attempt was made to establish the presence of the carrier state.

On the basis of the high mortality produced upon intraperitoneal inoculation into guinea pigs and intramuscular and subcutaneous inoculation into mice and its toxicity for chickens, the agent can be assumed to be similar to the virus of Louisiana pneumonitis.

As yet, there has been no opportunity to perform the type of serum neutralization test described by Hilleman(10), nor to perform the toxin neutralization test(3,11) which might make the specific strain identification more definite.

It is of interest to note that Olson and associates suspected that some animal reservoir may have initiated the epidemic which they investigated in humans, but they found no such reservoir. The first known case in the 1943 epidemic was in the wife of a trapper living in an area with an abundant wild bird population, and could have been contracted through close contact with infected birds. It may also be significant that a potentially dangerous agent has been shown to be active currently among birds. Further work may throw additional light on the significance of these findings in the epidemiology of this type of human respiratory infection.

Summary. A virus has been isolated from 2 snowy egrets. This virus has been identified by histological and serological tests as a member of the psittacosis-lymphogranuloma group. It closely resembles the Louisiana pneumonitis (Borg) virus in host range infectivity studies.

† This virus, sometimes referred to as the Borg strain, was kindly supplied by Dr. Dorland Davis of the National Institutes of Health.

‡ A standard laboratory strain of psittacosis, originally isolated by Dr. K. F. Meyer from a parakeet. This strain was also supplied by Dr. Dorland Davis.

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Metabolism of Fructose by the Liver of Diabetic and Non-Diabetic Subjects.* (19186)

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The metabolic effects of fructose administered intravenously to man have been studied in this laboratory recently(1). During the period of infusion, fructose was removed at the same rapid rate from the peripheral venous blood of both normal subjects and diabetic patients deprived of insulin. The concentration of blood pyruvic acid rose markedly in both groups of subjects. There was an increase in the blood glucose level of most of the normal subjects and of all of the diabetic patients investigated; in the latter group the glucose rise was of greater magnitude than in the normal group. Other investigators have demonstrated the participation of the liver in fructose metabolism in experiments with tissue slices or homogenates and with intact animals(2-8). Accordingly, hepatic vein catheterization studies were performed to evaluate the role of the liver in fructose metabolism in man.

Experimental procedure and methods. The subjects were 3 diabetic and 3 non-diabetic patients. In 2 of the cases, the diabetes mellitus was mild and easily regulated. Subject M.M. had had severe, labile diabetes mellitus for 23 years; she was in diabetic ketosis during the test and her CO_2 combining power was 30 volumes per 100 ml at the end of the test. None of the patients had evidence of hepatic disease. For 3 days prior to the test each patient received B complex capsules which provided at least 20 mg of thiamine daily; these were given to eliminate any possible effect of a sub-clinical thiamine deficiency on pyruvate metabolism. The diabetic patients were given only regular insulin for 3 days before the test and none on the morning of the test; therefore, no insulin effect was present at the time of the procedure. The tests were performed after an overnight fast. Pre-medication consisted of 0.032 g of codeine phosphate and either 0.2 g of phenobarbital or 0.1 g of seconal. A radiopaque catheter was inserted into a superficial arm vein and its tip was guided under fluoroscopic control into a right hepatic vein. Following a control period, 1.0 g of fructose‡ per kg was administered intravenously in a 10% solution in approximately one hour. Specimens of blood were obtained simultaneously from the hepatic vein and a peripheral artery during the control period; at approximately 30, 45, and 60 minutes after the beginning of the fructose infusion; and 30 minutes after the end of the infusion. These specimens were analyzed in duplicate for the following substances: Fructose(9); total hexose (Somogyi's

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TABLE I. Effects of Intravenous Fructose on Blood Concentrations and Net Splanchnic Assimilations of Fructose and Pyruvic Acid.

Date	Subject, age, wt, S.A.*	Time, min	Fructose: Artery, mg/100 ml	Fructose: Hepatic vein, mg/100 ml	Fructose: Net splanchnic assimilation, mg/min/m²	Pyruvic acid: Artery, mg/100 ml	Pyruvic acid: Hepatic vein, mg/100 ml	Pyruvic acid: Net splanchnic assimilation, mg/min/m²
				A. Non-diabetic subjects				
5/15	P.	0	2	2	0	.7	.6	1
	39 yr	41†	90	49	250	2.9	4.4	—9.2
	72 kg	66†	82	48	320	3.3	4.6	—12
	1.85 m²	97	23	19	49	2	2.6	—7.4
5/29	M.	0	2	3	—6	.9	.7	1.2
	59 yr	31†	170	132	300	2.4	3	—4.7
	57 kg	41†	130	87	330			
	1.66 m²	46†	108	64	400	2.6	3.9	—12
		81	26	20	43	2	2.6	—4.3
9/17	F.	0	1	1	0	.8	1.3	—4
	41 yr	35†	94	46	380	3.2	6	—22
	73 kg	48†	99	54	360	3.1	5.3	—18
	1.71 m²	60†	110	59	370	3.4	5.8	—18
		92	21	15	30	2.5	3.1	—3
				B. Diabetic subjects				
5/22	R.	0	2	2	0	1.2	.8	1
	52 yr	31†	128	71	160	3.5	5.7	—6.2
	68 kg	55†	150	76	240	3.9	6.9	—9.6
	1.66 m²	91	34	28	17	3.4	4.5	—3.1
6/5	B.	0	2	1	8	.9	.8	.8
	33 yr	30†	85	32	390	2.8	4.4	—12
	70 kg	45†	86	42	360			
	1.82 m²	60†	72	42	250	4	6	—17
		90	20	16	36	2.7	2.7	0
9/24	M.M.	0	2	2	0	1	.8	2
	28 yr	30†	96	54	390	1.3	1.1	1.8
	61 kg	46†	84	52	320	1.7	.9	8
	1.60 m²	54†	84	52	310	1.4	1.1	2.9
		85	24	21	39	1.7	.7	13

* Surface area. † During fructose infusion.

iodometric method for glucose)(10); pyruvic acid(11); citric acid(12,13); and, in some cases, malic acid(14) and lactic acid(15). Glucose was computed as the difference between total hexose and fructose. There was no interference with the fructose determination by blood glucose levels up to 300 mg per 100 ml; corrections were made for concentrations above 300 mg per 100 ml. The hepatic blood flow was estimated at appropriate intervals by the BSP technic(16,17). The estimated net splanchnic assimilation of a substance was calculated by multiplying its arterial-hepatic venous concentration difference by the estimated hepatic blood flow; the results were expressed in milligrams per minute per square meter of body surface. A positive value indicated retention of the substance by the splanchnic system, and a negative value indicated that the substance was being released from the system. It was assumed that the metabolic changes which took place in the splanchnic system occurred chiefly in the liver.

Results. Results of these studies are recorded in Tables I and II. Large amounts of fructose were retained by the liver in both diabetic patients deprived of insulin and in non-diabetic subjects. It was estimated that the liver removed approximately one-third of the administered fructose during the period of the infusion; during the 30 minutes after the infusion, approximately another one-sixth of the infused fructose was retained by the liver. During the *control period* the liver removed small amounts of pyruvic acid from the blood in 5 out of 6 cases; in the sixth case a small amount of pyruvic acid was released. When fructose was administered, there was a large output of pyruvic acid from

TABLE II. Effects of Intravenous Fructose on Blood Concentrations and Net Splanchnic Assimilation of Glucose.

Subject	Time, min	Glucose: Artery mg/100 ml	Glucose: Hepatic vein mg/100 ml	Glucose: Net splanchnic assimilation mg/min/m²
		A. Non-diabetic subjects		
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	[illegible]	[illegible]	[illegible]	[illegible]
	[illegible]	[illegible]	[illegible]	[illegible]
	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	[illegible]	[illegible]	[illegible]	[illegible]
	[illegible]	[illegible]	[illegible]	[illegible]
	[illegible]	[illegible]	[illegible]	[illegible]
	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	[illegible]	[illegible]	[illegible]	[illegible]
	[illegible]	[illegible]	[illegible]	[illegible]
	[illegible]	[illegible]	[illegible]	[illegible]
	[illegible]	[illegible]	[illegible]	[illegible]
		B. Diabetic subjects		
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	[illegible]	[illegible]	[illegible]	[illegible]
	55*	205	[illegible]	[illegible]
	91	201	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	30*	144	[illegible]	[illegible]
	45*	[illegible]	[illegible]	[illegible]
	[illegible]	[illegible]	[illegible]	[illegible]
	[illegible]	[illegible]	[illegible]	[illegible]
	[illegible]	[illegible]	[illegible]	[illegible]
M.M.	0	386	[illegible]	[illegible]
	[illegible]	[illegible]	[illegible]	[illegible]
	[illegible]	[illegible]	[illegible]	[illegible]
	[illegible]	[illegible]	[illegible]	[illegible]
	[illegible]	[illegible]	[illegible]	[illegible]
	[illegible]	[illegible]	[illegible]	[illegible]

* During fructose infusion.

the liver in all cases except patient M.M., who had severe diabetes mellitus and was in ketosis during the test. In the latter case the liver continued to remove pyruvic acid from the blood during the fructose infusion and removed an increased amount in the post-infusion period; in spite of the continued uptake of pyruvic acid by the liver, the arterial level of pyruvic acid increased. *Lactic acid* levels were determined in 4 cases. The changes in lactic acid usually paralleled those in pyruvic acid, but the magnitude of the lactic acid changes was greater. In one diabetic and 2 non-diabetic subjects the liver released lactic acid during the fructose infusion. In the fourth case (M.M., a diabetic patient in ketosis), lactic acid was retained by the liver in the control period, neither retained nor released at the mid-point of the infusion, and retained in increasing amounts in the subsequent periods; the arterial level of lactic acid showed a marked increase. In the 3 cases in which pyruvic and lactic acid were released from the liver, approximately one-fourth of the fructose uptake could be accounted for by the hepatic output of these two acids. In all 6 cases the liver released glucose during the control period. During fructose administration there was an increase in the hepatic glucose output in 3 cases (A.M., a non-diabetic subject, and E.R. and W.B., diabetic patients), and a decrease in the other 3 cases (non-diabetic subjects B.P. and L.F. and diabetic patient M.M.). There was an increase in the arterial level of glucose during the fructose infusion in all 6 cases.

Small quantities of citric acid were retained by the liver during the control period, and the administration of fructose did not produce any significant change in the splanchnic balance of

this acid. In the 3 cases in which blood malic acid levels were determined, the arterial-hepatic venous concentration differences were too small to be significant both in the control period and after fructose infusion.

Discussion. Other investigators have studied the metabolism of fructose in liver slices or homogenates and in the liver of intact animals. Their results indicate that fructose may be (a) phosphorylated under the influence of a hexokinase separate from that for glucose (2,3); (b) converted to glycogen(4); (c) transformed to glucose(3,5); (d) metabolized to lactic acid(6,7); (e) converted to fatty acids(8); or (f) oxidized completely to CO_2 (8). Our studies furnish evidence of the occurrence of (d) in the human liver *in vivo*; it is probable that hepatic production of lactic and pyruvic acid plays an important role in the rise in the concentration of these substances in the peripheral blood after fructose administration. Transformation of fructose to glucose may also have occurred in some of the cases; but in only 3 instances could increased hepatic glucose production have been responsible for an elevation in the peripheral blood level of glucose; other mechanisms must be invoked to account for the glucose rise in the other cases. Our technics of investigation do not permit an evaluation of the relative importance of the other possible routes of fructose disposal. Stein, Cori, and Cori(2) concluded from tissue studies that fructose utilization by the liver was not inhibited by glucose; this conclusion is apparently applicable also to the human liver *in vivo*. The depletion of liver glycogen which occurs in diabetic acidosis(18) may explain the divergent results obtained in case M.M. In the absence of normal hepatic glycogen stores, fructose may be converted to glycogen or glucose rather than to pyruvic and lactic acid. The rise in pyruvic and lactic acid concentrations in the arterial blood in this case must have been of extra-hepatic origin. Although the estimation of hepatic blood flow is admittedly inexact, the determination was used in this study to obtain an approximate value for the net splanchnic assimilation of certain substances. The conclusion that a substance was being retained by the liver or released from that organ was based only on the relationship of its concentrations in arterial and hepatic venous blood, and did not depend upon the magnitude of the hepatic blood flow. While errors in the estimation of hepatic blood flow would result in an erroneous calculation of the quantities of substances involved, the possible range of error does not appear to be great enough to invalidate any of our conclusions.

Summary and conclusions. The metabolism of fructose in the liver was investigated by hepatic vein catheterization studies in 3 diabetic patients deprived of insulin and in 3 non-diabetic subjects. (1) There was a large hepatic uptake of fructose during the period of intravenous administration in both diabetic and non-diabetic subjects. (2) In all but one case there was a large hepatic output of pyruvic and lactic acid during the fructose infusion. The liver of one diabetic patient in ketosis continued to remove pyruvic and lactic acid from the blood; hepatic glycogen depletion may have accounted for this divergent result. (3) In half the cases (2 diabetic patients and 1 non-diabetic subject) the output of glucose by the liver was increased during fructose administration. (4) In the absence of ketosis, the liver of the diabetic subject without insulin, therefore, metabolized fructose in a manner similar to that of the liver of the non-diabetic individual. The presence of ketosis, however, was accompanied by a decreased output of pyruvic and lactic acid, despite a normal uptake of fructose.

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Effect of Rabies Street Virus in the Cotton Rat and the Swiss Albino Mouse. (19187)

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The rabies virus strain V 308 used in the present study was isolated in Swiss albino mice (21-day-old Webster Carworth strain) from a dog brain sent to this laboratory for a routine examination for rabies. The history of this strain is given in a previous paper which dealt with electron microscope studies of Negri bodies(1). The strain was pathogenic for 2 species of bats(2). Furious rabies was produced in the Syrian hamster by various routes of inoculation with this strain (3).

Methods. Each of four 3-week-old cotton rats (*Sigmodon hispidus*) was inoculated intracerebrally with 0.03 cc of a 10% virus-bearing brain suspension of the 2nd mouse passage which titrated 10^{-4} in mice. Five days after injection the cotton rats showed tremors and paralysis, but no evidence of furious rabies was noted. The brains of 2 paralyzed cotton rats were removed aseptically. Touch preparations from the Ammon's horns of these brains were stained with Seller's stain(4) and upon examination under an optical microscope were found to contain Negri bodies. A 10% suspension in physiological saline was made from the 2 brains. This material was used to initiate the present study in cotton rats and Swiss albino mice. By intracerebral inoculation it titrated $10^{-4.5}$ in cotton rats and $10^{-4.2}$ in mice. Diagnosis of rabies was made on the following factors: presence of symptoms of rabies in the rat or mouse and demonstration of Negri bodies in the Ammon's horn of the brain of the animal showing symptoms. The presence of Negri bodies was determined by staining touch preparations of the Ammon's horn with Seller's stain and examining the slides with the optical microscope. Twenty-seven healthy cotton rats (21 days old) were divided into 9 groups of 3 animals each. The 3 rats of each group were administered the virus by one of the following methods: intracerebral, intraperitoneal, intradermal, intramuscular, intralingual, and intracardial injection and by oral, rectal, and intranasal instillation. In all routes of exposure the inoculum consisted of 0.1 cc of the above described suspension, except in the intracerebral group where the inoculum was 0.03 cc. Twenty-seven healthy Swiss mice of the same age were divided into 9 groups of 3 each and were treated in a like manner. After inoculation all groups were observed daily for symptoms of rabies. After symptoms of rabies appeared and the animal became paralyzed or moribund, it was sacrificed and the brain removed and examined for the presence of Negri bodies. Some animals died during the night and the brains of these

TABLE I. Response of Cotton Rats and Swiss Mice to Rabies Virus. All showed symptoms.*

Min-max incubation period† (in days)	
Cotton rats	Swiss mice
7-8	9-10
7-8	7-8
9-11	7-8
7-8	7-8
12-14	10-11
8-11	7-8
5-6	6-7
5-10	8-11
—	14

* Except cotton rats by intranasal inoculation.
† Incubation period—time between exposure to virus and onset of symptoms.

dead rats or mice were also examined for Negri bodies.

Results and discussion. Table I shows the response of cotton rats and Swiss mice injected with rabies virus by different routes of exposure. All rats and mice exposed, except the cotton rat group exposed by intranasal instillation, died from the disease or were sacrificed when symptoms appeared.

The intranasal group of rats was held 30 days without showing symptoms. They were then sacrificed and touch preparations of their brains examined. No Negri bodies were seen.

No cotton rats or Swiss mice showed symptoms of furious rabies as found in Syrian hamsters infected with this strain. The incubation period in rats and mice was between 5 and 14 days by these various routes of exposure. The cotton rats were as susceptible as the Swiss mice to this V 308 strain of rabies virus. This strain of mice was the one recommended by Webster as being especially susceptible to neurotropic viruses(5).

Summary. A strain of rabies isolated from a dog brain and passaged intracerebrally 2 times in Swiss albino mice and one time in cotton rats has been successfully transmitted to the same rodents by the following routes of exposure: intracerebral, intraperitoneal, intradermal, intramuscular, intracardial, intralingual, oral and rectal. The cotton rats and Swiss mice were equally responsive to this strain by the above routes of exposure. Mice were susceptible to intranasal instillation but cotton rats exposed by this method remained symptom free during a 30-day observation period and there was no evidence of Negri bodies when their brains were examined at the end of that time. This strain of virus, which had produced furious rabies in Syrian hamsters in a previous experiment, produced the dumb form of rabies in the cotton rat and Swiss albino mice.

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An Adsorption Technique for Partial Purification of Japanese Encephalitis Virus in Chick-Embryo Tissue Suspensions. (19188)

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Crude suspensions of chick-embryo tissues infected with the virus of Japanese encephalitis contain a miscellany of tissue materials differing widely in particle size, composition, and physical properties. As a preliminary step toward the isolation of the virus it would be desirable to utilize some simple method to remove most of the extraneous material, leaving a partially purified and considerably more uniform suspension of unimpaired infectivity.

A certain amount of the extraneous material can be removed by differential centrifugation.

The coarse particles which are much larger than the virus can be separated readily in this way and in some instances the tissue material of smaller size can be separated by sedimentation of the virus particle itself. The latter technic can be used with chick-embryo suspensions infected with Eastern, Western or Venezuelan equine encephalomyelitis(1) but has not proved effective in separating Japanese encephalitis virus. The centrifugal separation of Japanese encephalitis virus from infected mouse-brain tissues has also failed to be practicable(2).

In this laboratory experiments have been conducted to explore the possibility of adsorption as a means of removing the smaller tissue constituents of a crude chick-embryo suspension. The adsorbent which has been used is Attaclay SF,* a naturally active, highly sorptive clay which is available in large quantities as a dry finely-divided powder. The clay is an essentially inert, neutral material with an average particle diameter of 400 to 600 mμ. The exceedingly small particle size of this material suggested that it would be a poor adsorbent for the virus particles and would tend to reject them in favor of the smaller constituents of the suspension.

Methods. The virus used in these experiments was the Nakayama strain of Japanese encephalitis. Tissue suspensions were prepared from normal or infected chick embryos homogenized in a Waring Blendor with sufficient phosphate-buffered physiological saline (pH 7.8) to make 20% suspensions. Coarse tissue fragments were removed by centrifugation at 2000 rpm for 10 minutes.

Samples of the supernatant fluid from this centrifugation were treated with various volumes of dry, sterile (autoclaved) Attaclay SF. The volumes used were measured in graduated glass cylinders with tapping to pack the clay particles reproducibly. In each instance the amount of clay was specified as ml of clay per 100 ml of fluid. The fluid and clay were placed in a stoppered centrifuge tube of such size that the volume of the contents was about half the total capacity of the tube. Tube and contents were shaken vigorously by hand for 5 minutes. During the shaking process the temperature of the fluid ranged from 10 to 15°C and within this range did not appear to be a critical factor. After this shaking the tubes were cooled to 2 to 4°C and centrifuged for 15 minutes at 3200 rpm. The clarified supernatant fluid was decanted and if necessary, centrifuged again to remove the last traces of the adsorbent. Before and after treatment with the clay the suspensions were sampled for infectivity titrations and for total nitrogen assay by the micro-Kjeldahl method. Infectivity was determined by the intracerebral inoculation of 0.03 ml doses of suspension using groups of 5 Bagg strain, Swiss albino mice for each serial 10-fold dilution. The infective titer was expressed as —log LD_{50}.

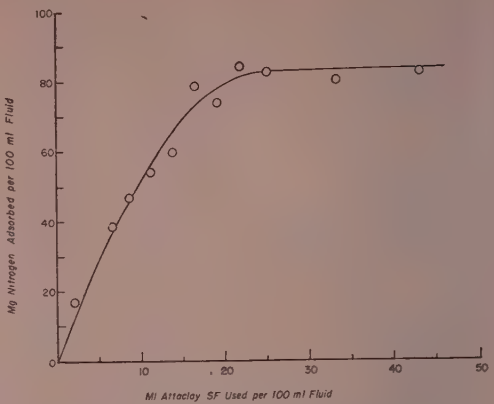

FIG. 1. Adsorption of nitrogenous material from 20% normal chick embryo suspension using varying amounts of Attaclay SF.

Results. The relationship between the quantity of adsorbent used and the amount of nitrogenous material removed from 20% suspensions of normal chick embryo tissue is illustrated in Fig. 1. Twenty ml of clay per 100 ml of fluid appeared to be the optimal amount of adsorbent. This amount removed almost all of the nitrogenous material which could be adsorbed and larger quantities of adsorbent were not appreciably more effective. It was noted that the pink color in the crude suspensions due to hemoglobin was usually removed by as little as 10 ml of clay per 100 ml of fluid.

The effect of the clay treatment upon the

* Supplied by courtesy of Attapulgus Clay Co., Philadelphia, Penna.

TABLE I. Infective Titer and Total Nitrogen Content of Infected 20% Chick-Embryo Tissue Suspensions Before and After Adsorption with Attaclay SF.*

Trial No.	Infective titer ($-\log LD_{50}$) Before	After	Total N (mg/ml) Before	After	% N reduction
1	7.7	6.9	.93	.20	79
2	6.8	6.6	1.01	.24	76
3	8	7.2	1.11	.29	74
4	6.5	6.6	1.36	.45	67
5	5.5	5.2	1.07	.27	75
6	6.2	6.6	1.39	.40	71
7	6.6	7	1.13	.29	74

* 20 ml of dry packed clay used for each 100 ml of tissue suspension.

infectivity and the nitrogen content of the suspensions is summarized in Table I. The data show that it is possible by this method to remove about 75% of the nitrogen content of a crude virus suspension without serious loss of infectivity. It should be pointed out that the figure expressing the infective titer of a virus preparation is inherently of a low order of accuracy (± 0.3 log unit). The apparent losses of infectivity in Trials 1 and 3 do not appear significant when considered in context with the apparent gains in Trials 6 and 7. Some of the infective particles are undoubtedly lost in the adsorption process and in the subsequent centrifugation. However, the loss is usually not sufficient to result in a significant decrease in infective titer.

The simplicity and convenience of the technic described suggest that it may have a useful application as a preliminary step in virus purification and in the preparation of vaccines and diagnostic antigens.

Summary. Japanese encephalitis virus in 20% suspensions of infected chick-embryo tissues may be partially purified by adsorption of inert material on particles of Attaclay SF, a naturally-occurring sorptive clay of exceedingly small pore diameter. Removal of about 75% of the nitrogen content of a crude virus suspension may be accomplished without a prohibitive loss of infectivity.

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Excretion of Amino Acids in Cystinuria. (19189)

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The precise nature of the metabolic disturbance responsible for cystinuria remains a matter of conjecture. At one time the disease was considered by many to be linked exclusively to aberrations in the metabolism of the sulfur containing amino acids. Numerous reports have appeared, however, indicating that amino acids other than cystine may be excreted in abnormal amounts by the cystinuric. References to the early literature on the subject may be found in Garrod(1). More recently, Yeh, Frankl, Dunn, Parker, Hughes and Gyorgy(2) employed microbiological assay to analyze the urine of an 8-year-old cystinuric girl and observed a consistent and unusually high level of lysine and arginine excretion. Since then Dent(3) and Dent and Rose(4) have employed paper chromatography to investigate the amino acid content of a number of cystinuric urines. Although the paper chromatographic method is not quantitative, Dent and Rose were led to

the conclusion that lysine, not cystine, was the amino acid excreted in largest amounts in cystinuria, and that the quantity of arginine excreted was also markedly elevated in most cases. It became of interest, therefore, to examine the amino acid content of cystinuric urines employing the recently developed quantitative chromatographic technics which employ columns of the ion exchange resin Dowex-50(5).

Cases. The cases studied had all been diagnosed previously as cystinurics. It is a pleasure to acknowledge with thanks the help of Dr. Robert F. Watson of the New York Hospital, Dr. George A. Perera of College of Physicians and Surgeons, Columbia University, and Dr. Frank L. Horsfall, Jr., of the Rockefeller Institute, in securing the urine specimens. A brief summary of the pertinent aspects of the case histories follows: *Case 1.* A 29-year-old, married, white male, weight 150 lbs. Has suffered intermittently from bilateral renal colic, passage of stones, and surgical removal of stones since the age of 17. *Case 2.* A 58-year-old, married, white female, weight 192 lbs. The patient has had intermittent renal colic and has passed stones. Right nephrectomy was performed at age 31. *Case 3.* A 29-year-old, single, white female, weight 133 lbs; suffered intermittently from renal colic; passed a right renal calculus 3 years ago. *Case 4.* A 49-year-old, single, white male, weight 202 lbs; suffered from left renal colic; passed a stone at age 36. *Case 5.* A 31-year-old, married, white female, weight 115 lbs. Has a long history of renal complaints including bilateral calculi and pyelonephritis.

Methods. The urine samples chromatographed were withdrawn from 24-hour specimens. The subjects were leading a normal life at home during the period of the urine collection, and no attempt was made to control their diet. The bottle furnished each individual for collection of the urine contained 6 cc of chloroform and 6 cc of toluene as a preservative. The specimens all arrived in the laboratory within a few hours of the close of the collection period, and were stored thereafter at 4°C. The first chromatograms (the 100 cm columns referred to below) were usually run immediately. The 15 cm columns were run 1 to 2 weeks later. Two urine samples (Cases 3 and 4) deposited a precipitate before samples could be withdrawn for the 100 cm columns. These specimens were stirred vigorously and 25 cc poured off rapidly into a graduate and enough N HCl (about 2.5 cc, predetermined on a separate aliquot) was added to bring the pH to 2.3 to 2.5. Although this procedure served to dissolve most of the precipitate, it is possible that some cystine, if it came out of solution, did not redissolve. The cystine figures for Cases 3 and 4 may be slightly low on this account. Prior to chromatography on the 100 cm columns, all urine samples (about 3 cc in volume) were brought to pH 2.3 to 2.5 with N HCl. The chromatograms were run in the manner recommended(5) for urine, no desalting being required. In order to increase the resolution in the basic amino acid range, a phosphate buffer of pH 7.5 (prepared by mixing 200 cc of 0.1 M Na_2HPO_4 and 15 cc of 0.2 M $NaH_2PO_4 \cdot H_2O$) was interposed for 180 cc between the pH 6.7 and pH 8.3 buffers. The pH 8.3, 9.2, and 11.0 buffers each were run for 100 cc. For the quantitative determination of the basic amino acids, 1 cc of urine was chromatographed on the 15 cm columns of Dowex-50 recommended heretofore(5). At the close of each of these latter chromatograms, fractions of the 0.2 N NaOH employed to clean out the columns were collected and analyzed by the ninhydrin method. In this manner it was established that only very small amounts of tenaciously held basic substances remained on the columns. A single 100 cm and a single 15 cm chromatogram were run on each sample of urine.

Results. Those amino acids found in grossly abnormal amounts in cystinuric urine are given in Table I. It is clear that arginine, lysine, cystine, and also ornithine are excreted by cystinurics in 50 to 100 fold the amounts found for normal subjects. In addition, the isoleucine level in cystinuric urine is elevated by about twofold, whereas taurine is excreted in unusually small quantities, about ⅓ or less of the normal. By virtue of the chromatographic method of analysis employed, it is possible to state that the quantities of all the

TABLE I. Excretion of Amino Acids by Five Cystinuries.

Amino acid	Excretion in g per diem					Normal range*	Relative molar excretion Ornithine chosen as 1				
	Case 1	2	3	4	5		Case 1	2	3	4	5
Ornithine‡§	.42	.18	.36	.50	.42	None	1	1	1	1	1
Cystine	.97	.42	.82†	.74†	.70	.01 -.03	1.3	1.3	1.3†	.83†	.91
Arginine	1.24	.55	.92	.77	.67	0 -.02	2.3	2.3	2	1.2	1.2
Lysine‡	2.30	1	1.98	2.38	1.35	.01 -.05	4.9	5	5.1	4.4	2.9
Isoleucine	.057	.038	.043	.065	.064	.015-.03					
Taurine	.05	.008	.062	.086	.052	.1-.3					

* Unpublished data.

† These values may be low.

‡ Ornithine and lysine yield completely separated but neighboring peaks on the 100 cm Dowex-50 columns. Although quantitative recoveries of the basic amino acids are not obtained under these conditions, the relative amounts of ornithine and lysine can be determined. A value for the sum, ornithine + lysine, can be obtained from the short 15 cm column which, however, does not separate these two amino acids. Combining the data from the 15 cm and 100 cm columns, it is readily possible to calculate the amount of both ornithine and lysine present.

§ Ornithine was also identified on the 100 cm columns by analyzing alternate fractions of the ornithine peaks by the color reaction of Chinard in the manner already indicated (6).

other ninhydrin positive substances in these cystinuric urines are within the normal range, and furthermore, that no unusual ninhydrin positive substances (cadaverine or putrescine, for example) (1,4) occur in appreciable amounts. In one instance (Case 1), a second sample of urine collected some weeks later gave results agreeing to within ± 5 to 20% with those given in Table I. Upon hydrolysis of a single urine sample (Case 1) with boiling 6 N HCl for 16 hours, the values for arginine, ornithine, lysine, taurine, and isoleucine agreed to ± 10% with those obtained before hydrolysis. The cystine figure after hydrolysis was substantially lower, doubtless as a result of the destruction of cystine during hydrolysis.

Discussion. Dent and Rose (4) have proposed that the diagnosis of cystinuria be limited to include only those individuals who excrete large quantities of cystine, lysine, and arginine. The data reported in this communication suggest that the pattern of the amino acid excretion in cystinuria is even more definitive, and is characterized by a very excessive excretion of cystine, lysine, arginine and ornithine, a moderately excessive isoleucine excretion, and a markedly diminished output of taurine.

The data given in the right hand half of Table I show that the relative molar amounts of cystine, arginine, ornithine, and lysine excreted are quite similar from one subject to the next. The similarity of these figures is all the more striking in view of the heterogeneous nature of the case material, and the fact that no attempt at dietary control whatsoever was attempted at any time during or before the period when the urine specimens were collected. Dent and Rose (4) believe that cystinuria is the result of a physiological defect in the kidney tubule, and consists in a failure to reabsorb some part of the basic amino acids and cystine. Previous metabolic studies with the sulfur amino acids (7-9), coupled with the observations reported here, would appear to be more compatible with the working hypothesis that cystinuria is a metabolic defect, enzymatic in nature, which affects simultaneously at some site or sites (probably the kidney) some phase of the metabolism of arginine, ornithine, lysine, and the sulfur amino acids, and perhaps also isoleucine and taurine.

Summary. The amino acid content of the urine of five cystinurics has been determined employing chromatography on columns of the ion exchange resin, Dowex-50. In every case the amount of arginine, lysine, ornithine, and cystine was found to be from 50 to 100 fold the normal level. The quantity of isoleucine was about twice the normal, whereas the taurine output was diminished to about ⅓ or less of normal. All other ninhydrin positive constituents were in the normal range. It is particularly striking that the relative molar

excretion of ornithine, cystine, arginine, and lysine, which was found to be about 1:1:2:4, is quite similar from one subject to the next despite the haphazard nature of the case material and the absence of any dietary control. The results suggest the hypothesis that cystinuria involves an enzymatic defect which affects simultaneously at some site or sites (probably the kidney) some phase of the metabolism of arginine, lysine, ornithine, and the sulfur amino acids, and perhaps also isoleucine and taurine.

It is a pleasure to acknowledge the expert laboratory assistance of Mrs. Gertrude C. Carey.

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The Screening of Antiulcer Drugs by a Quantal Procedure. (19190)

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The pyloric ligation method of Shay(1) which has won universal acceptance has made it possible to solve many problems of the pathogenesis of gastric ulcer(2-4). The Shay rat, however, has been used only occasionally for evaluating the antiulcer effect of drugs: antacids(2,3), Thephorin(6), tetraethylammonium(7), phenothiazine derivatives(8), and urinary extracts(4,9).

Moreover, studies of the activity of atropine have been quite contradictory. Protection has been noted with 250 mg/kg subcutaneously (2); 10 mg kg(6); 1.5-3 mg/kg(10); whereas, no protection has been observed with 100 mg kg(8). A systematic investigation of compounds reported clinically effective in peptic ulcer therefore seems warranted. For that purpose a quantal procedure has been devised.

Material. Twelve drugs were tested. A. Anticholinergic agents: 1. atropine (sulfate) USP; 2. trihexyphenidyl, *Artane.* B. Pure ganglionic blocking agents: 1. pentamethonium bromide; 2. hexamethonium bromide; 3. tetraethylammonium bromide; 4. tetraethylammonium chloride. C. Ganglionic blocking-anticholinergic agents: 1. B-diethylaminoethyl-xanthene-9-carboxylate methobromide, Methantheline Bromide, *Banthine;* 2. 10 - (B - diethylaminoethyl) - phenothiazine (hydrochloride) *Diparcol.* D. Antihistaminic agents: 1. tripelennamine hydrochloride USP, *Pyribenzamine;* 2. diphenhydramine hydrochloride USP, *Benadryl;* 3. phenindamine hydrogen tartrate, *Thephorin;* 4. Chlorothen Citrate, *Tagathen.* 580 male albino Wistar rats maintained on a diet of Wayne "Rat Blox" were used; those weighing 180-250 g were starved for 72 hours before the ligation, while younger rats weighing 125-180 g were starved for 48 hours only. *Method.* The original Shay rat method(1) was used. However, whereas previous authors used as criteria the decrease of gastric acidity and volume, together with the pepsin concentration and a system of scoring the severity of ulcerations, the test selected in our assays concerns only the appearance of ulcerative lesions in the rumen of the rat ob-

TABLE I. Average Protective Dose of Drugs Against Ulcer Formation in the Shay Rat. Single subcutaneous administration; rats 125-250 g; sacrificed 17-19 hr after ligation.

Drug	No. rats	ED_{50}, mg/kg	19/20 confidence limits	P	Slope	λ	LD_{50}
Control	78						
Atropine (SO_4)	53	11	7.3–16.5	.91	2.8	.35	
Artane	34	7.0	3.9–12.5	1	2.6	.38	
Diparcol	12	>200					200
Banthine	44	5.3	3.5–7.9	.97	2.0	.52	
Pentamethonium	12	>200					
Hexamethonium	18	>200					200
Tetraethylammonium (Br.)	6	>200					200
" (Cl.)	18	>200					<200
Pyribenzamine	26	> 75					75*
Benadryl	23	>300					400
Thephorin	51	98	75–125		1.3		*
Tagathen	20	>150					

* Convulsion at 100 mg/kg.

served under a 10x binocular microscope. In order to modify the graded response procedure previously used into a quantal one, the 50% protective dose (ED_{50}) was determined. The present method involves treating groups of Shay rats with graded doses of antiulcer compounds and evaluating the probit of each group which fails to show ulcers following pyloric ligation. From the data so obtained a regression line is constructed and the ED_{50} evaluated and compared with the ED_{50} of atropine used as standard. In designing such a quantal response, the selection of the period of ligation which gives 100% ulcer in controls is essential. In preliminary experiments the 17-19 hours period selected by Shay for adult rats (180-250 g) was used. The period was decreased to 10 hours in a second group of experiments.

Results. 1. *Adult rats injected subcutaneously at the time of ligation and sacrificed 17-19 hours later.* Table I summarizes the results in Shay rats of the average protective dose (ED_{50}); the 19/20 confidence limits calculated according to Litchfield and Wilcoxon; the probability factor (P) according to Fisher; the slope of the curve; the standard deviation of the logarithms of the individual effective dose, and the reciprocal of the slope (λ). The comparison between the dose inducing side effects or toxic symptoms and the protective dose listed in Table I gives some information about the specificity of action of some drugs. Of interest is the increased toxicity of Pyribenzamine on the Shay rat as compared with data of the literature (11) and of our own experiments on normal rats.* Fig. 1 shows a linear relationship between the probit of protected rats and the logarithm of the dose of atropine, Banthine, Artane, and Thephorin. Neither antihistamine compounds such as Benadryl, Pyribenzamine, and Tagathen nor ganglionic blocking agents such as pentamethonium, hexamethonium, and tetraethylammonium were active unless doses causing side effects or toxic effects were used.

2. *Young rats injected subcutaneously both at the time of ligation and 5 hours later and sacrificed 10 hours after ligation.* Because of the ineffectiveness in the Shay rat of pentamethonium, hexamethonium, and tetraethylammonium, which are reported clinically active, it seemed important to study comparatively another method involving a shorter duration of ligation (10 hours) and using 2 injections, one at the time of ligation and the other 5 hours later. Preliminary experiments, however, showed that in such conditions in order to obtain 100% ulcers in controls, younger animals (50-100 g) should be used. This confirms the data of Shay who found that in 180 g rats ulcers were noted in only 72% of the controls. The results summarized in Table II show a significant increase in the

* Ether seems to potentiate the toxic effects of Pyribenzamine. 100 mg/kg Pyribenzamine subcutaneously induces 12/18 mortalities in unoperated male rats anesthetized with ether *versus* 2/18 in controls.

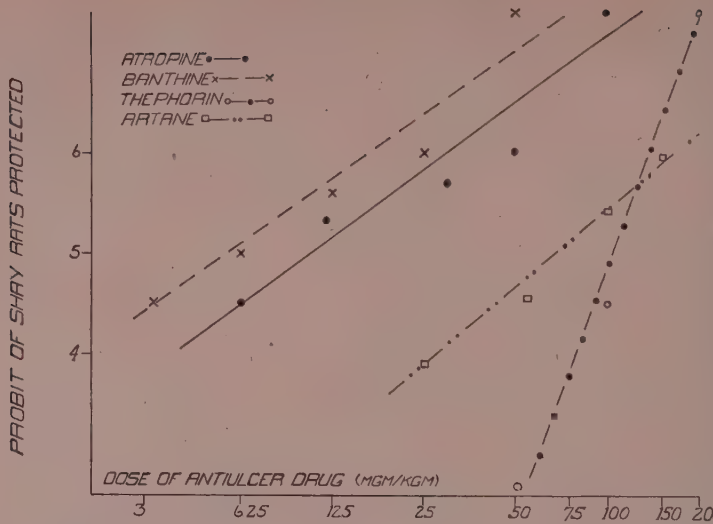

FIG. 1. Dose effect curve of atropine, Banthine, Artane and Thephorin. Abcissa: Dose of antiulcer (logarithmic scale). Ordinates: Probit of Shay rats protected against ulcers.

protection afforded by various drugs, especially short acting drugs: tetraethylammonium, hexamethonium, pentamethonium, and Diparcol.

3. *Adult rats submitted to oral administration and sacrificed 17-19 hours after ligation.* Because the oral route of administration is generally used for antiulcer compounds, comparative experiments were run following this route. Table III summarizes the effect of drugs given orally 1 hour before pyloric ligation, the animals being sacrificed 17-19 hours after ligation.

Discussion. The Shay rat has been found of value for the qualitative assay of antiulcer preparations, although its specificity has been debated(5). In order to give reproducible results, however, the procedure involves the laborious experimental effort of time-consuming tests and calculations. A modification into a quantal procedure is here described which is more rapid and more suitable for quantitative evaluations. The regression line when tested for linearity was found satisfactory and gave estimates of the potency of various clinically active drugs.

It was found essential to select 10 hours as the optimum time interval between the pyloric ligation and observation of the ulcerations. Table II shows a greater effect at 10 hours than 18 hours after injection. This result is of importance in the case of short acting drugs such as pentamethonium, hexamethonium, and tetraethylammonium. Of course, this interval period is not as practicable as 17 or 19 hours, which permits the running of experiments during the ordinary working schedule.

Contradictory results were reported concerning the antiulcer effect of histamine blocking agents. Our data indicate the inefficiency of Pyribenzamine, Benadryl, and Tagathen in the Shay rat and the slight activity of Thephorin, smaller than previously reported (6), observed by us only at a dose inducing central stimulation. This confirms the failure of these drugs to block histamine induced gastric secretion(12). Conversely, the effectiveness of atropinelike compounds in the Shay rat is in agreement with clinical experience.

Inasmuch as the ganglionic blocking action of Banthine is complicated by powerful anticholinergic effects, the specificity of its ganglionic site of action is questioned. Our results on the Shay rat reveal that Banthine falls in the class with atropine rather than with tetraethylammoninm; its ganglionic blocking effect is not, however, excluded.

Summary. (1) A modification of the pyloric ligation method of Shay into a *quantal*

TABLE II. Comparative Effect of Antiulcer Drugs According to Mode of Administration and Duration of Pyloric Ligation.

Drug	Total dose, mg/kg	Single inj., 17-19 hr ligation, % protected	No. rats	Double inj., 10 hr ligation, % protected	No. rats
Control			78		30
Atropine (SO_4)	22	72	53	100	6
Artane	100	66	34	100	6
Banthine	10.6	75	44	100	6
Pentamethonium	100	33	12	88	12
Hexamethonium	200	0	18	100	12
Tetraethylammonium (Br.)	100	0	6	50	12
" (Cl.)	100	0	18	66	18
Diparcol	200	0	12	100	6

TABLE III. Average Protective Dose of Drugs Against Ulcer Formation in the Shay Rat. Oral administration; rats 125-250 g; sacrificed 17-19 hr after ligation.

Drug	No. rats	ED_{50}, mg/kg	19/20 confidence limits	P	Slope	λ	LD_{50}
Control	36						
Atropine (SO_4)	29	70	47–105	.8	2.2	.45	
Banthine	18	140	110–255	1	1.6	.63	<400†
Thephorin	18	140	110–255	.5	1.5	.67	400*

* Convulsions at 100-400 mg. † Hemorrhage in the glandular area.

procedure is described. A linear relationship exists between the logarithm of the doses of antiulcer compound and the probits of Shay rats failing to show gastric ulcerations. (2) Anticholinergic agents such as atropine, Artane, and Banthine administered subcutaneously and orally show unequivocal protection against ulcerative lesions of Shay rats. (3) Short acting *pure* ganglionic blocking agents: hexamethonium, pentamethonium, tetraethylammonium afford significant protection only following a short duration of pyloric ligation and by using a fractionated injection. (4) No antihistamine agent seems to be effective against ulcer in the Shay rat. (5) The Shay rat is a valuable tool for the screening of antiulcer drugs.

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Coramine (Nikethamide) as Adjuvant to Atropine in Treatment of Poisoning by EPN (ethyl p-nitrophenyl thionobenzenephosphonate) (19191)

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There exists a number of organic phosphates (DFP, TEPP, parathion, EPN, *et al.*) having considerable economic and possible military importance. Since these compounds inhibit cholinesterase(1-3), they are extremely hazardous; the search for efficacious antidotes is important. At present, atropine because of its powerful parasympatholytic activity is the drug of choice in the treatment of poisoning by these agents(4).

It is believed that death following organophosphate poisoning results from respiratory failure(2). The administration of a respiratory stimulant, then, would seem to be advantageous. The purpose of this paper is to report the beneficial effect of a respiratory stimulant, coramine (nikethamide), when used as an antidote in poisoning by EPN.

Methods. Male and female albino rats were given graded doses of EPN in corn oil solution by stomach tube. Since the absorption of EPN may depend in part on the volume of oil given, approximately equal volumes were given each sex. All rats used were 2 to 3 months old; female rats weighed between 140-180 g and males between 200-240 g. To compensate for differences in body weights and in LD50's (Table I), the males received a 5% solution, the females a 1% solution. Atropine (90 mg/kg) and coramine (120 mg/kg) were given intraperitoneally (Table I) immediately after the stomach tubing procedure. The data represent twenty-four hour mortalities and were treated according to the method of Litchfield and Wilcoxen(5).

Results. It is evident from Table I that the simultaneous administration of coramine and atropine to *female* rats raised the LD50 ten times over that of EPN alone. Atropine when used by itself was capable of producing only a twofold increase in the LD50 of EPN.

The antidotal properties of the atropine-coramine combination were not so striking in EPN poisoned *males*. The LD50 in this instance was raised four times over that of EPN, whereas a 2-fold increase was evident when atropine was the sole antidote. Coramine alone was not an effective antidote and did not change the LD50 of EPN in either males or females.

The mechanism of action of the atropine-coramine combination is obscure. That it does not protect red blood cell cholinesterase levels was borne out by an experiment in which adult female rats were given a certainly lethal dose of EPN (60 mg/kg). Some were given atropine-coramine, some coramine alone, some no further treatment. All were sacrificed when the last-named group exhibited severe symptoms; blood was withdrawn by cardiac puncture. Individual samples tested by the method of Michel(6) showed no differences in the reduction of red blood cell cholinesterase activity regardless of treatment.

A number of the commonly used analeptics have also been given to EPN poisoned rats. These included metrazol, benzedrine (amphetamine), picrotoxin and caffeine; in each case the effect was *increased mortality*. There-

TABLE I. Dose-Effect Relationships Comparing the Antidotal Properties of Atropine and of Atropine-Coramine.

	Females			Males		
	LD_{50}, mg/kg	19/20 limits, mg/kg	No. of rats	LD_{50}, mg/kg	19/20 limits, mg/kg	No. of rats
EPN	6.7	7.1–6	71	33	39–28	95
EPN atropine	11.6	12.2–11	50	74	104–53	46
EPN atropine-coramine	71	86–59	80	120	216–67	53

fore, a word of warning is indicated: it is probable that these analeptics should *not* be given a desperately poisoned human patient.

Since all the observations reported herewith have been on rats, it is not implied that the findings can be directly applied to the treatment of human poisoning. In the rat tests, the antidotes were given simultaneously with the EPN, such conditions would rarely be possible in industrial or accidental human exposures.

Summary. The administration of coramine and atropine together was considerably more effective in the treatment of EPN poisoned rats (both male and female) than was atropine alone.

The authors acknowledge with pleasure the helpful criticism of Dr. John A. Zapp, Jr., Assistant Director of the Haskell Laboratory of Industrial Toxicology, E. I. duPont de Nemours and Co. We thank Ward D. Noyes who carried out the cholinesterase activity measurements.

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A Simple Method for the Determination of Urinary Amino Nitrogen.* (19192)

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Russell(1,2) adapted Folin's colorimetric technic(3) for the determination of blood amino nitrogen. According to Russell, this technic could not be used for urine because of interference by ammonia and uric acid. Therefore, it was the purpose of this investigation to adapt her procedure to the quantitative determination of urinary amino nitrogen.

Procedure. The reagents required, color reaction and technic were those described by Russell with the exception of Norit-A Decolorizing Carbon.

Removal of Ammonia: Volumes of urine ranging from 0.5 to 2.0 ml were pipetted into 200 ml Erlenmeyer flasks etched at the 100 ml mark. Approximately 75 ml of distilled water was added followed by 4 drops of 0.25% phenolphthalein in 95% ethanol. 0.1 N sodium hydroxide was added dropwise until the flask contents retained the pink color. This condition was reached at a pH between 9.0 and 9.5. The solution was boiled gently for 25 minutes. Base was added during the boiling process until the pink color no longer faded.

Removal of Uric Acid: After boiling off ammonia, the flask and its contents were cooled in an ice bath, and the solution acidified dropwise with 0.2 N hydrochloric acid until the pink color disappeared. Then one drop in excess was added. The solution was diluted to the 100 ml mark with distilled water and chilled in an ice bath for 10 minutes. Fifty mg of Norit-A Decolorizing Carbon was then added. The use of charcoal as an adsorptive agent for uric acid in urine was adapted from a procedure suggested by Bodansky(5).

After the addition of charcoal, the flask was stoppered, vigorously swirled for about a minute, and then allowed to stand at 5°C for a 2-hour period. At the end of this time, about 10 ml of the charcoal treated solution was transferred to a test tube and centri-

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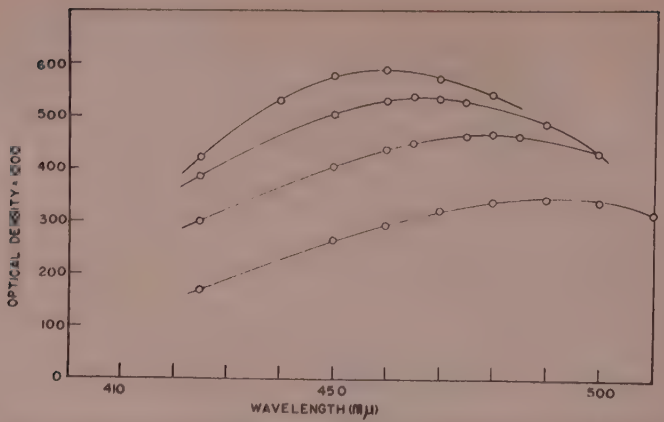

FIG. 1. Absorption spectra of urinary amino acid sodium-β-naphthoquinone-4-sulfonate complex.

fuged. After centrifugation, a 4 ml aliquot of the supernatant liquid was pipetted into the color development tube. The Russell color procedure followed from here.

Experimental. Absorption data were obtained for the color complex resulting when urinary amino compounds were coupled with the naphthoquinone reagent. Eight urine specimens were investigated in this manner.

In checking the technic for completeness of removal of ammonia, standard ammonium chloride solutions with an ammonium ion concentration approximately equal to that found in urine were subjected to the described boiling procedure, and analyzed for ammonia by the technic of Sobel(4).

In order to determine the optimum charcoal concentration for uric acid adsorption, carefully weighed quantities of charcoal were added to solutions of 3 mg per cent uric acid and known concentrations of standard glycine-glutamic acid mixture. The solutions were shaken, centrifuged, and the centrifugate analyzed for uric acid, by Brown's technic (6), and also for amino acid nitrogen. A glycine-glutamic acid standard curve was obtained for the range of 5 to 30 γ of amino acid nitrogen.

Results. Spectrophotometric absorption data were obtained for the urinary amino naphthoquinone complex in 8 different subjects. Representative spectra, showing the extremes in maximal absorption, are shown in Fig. 1. The lowest maximum absorption occurred at 460 mμ while the highest was at 490 mμ. The spectra gave a mean peak absorption of 475 mμ. In view of the broad absorption peaks observed and the mean absorption of 475 mμ, it was felt that the glycine-glutamic acid standard, which absorbed maximally at 470 mμ and was used by Russell for blood amino nitrogen, could be used for urinary determinations of amino nitrogen.

With this same standard solution, an essentially linear relationship between concentration and absorption was obtained for amino nitrogen concentrations within the range of 5 to 30 γ (Fig. 2).

Determinations for ammonia in boiled

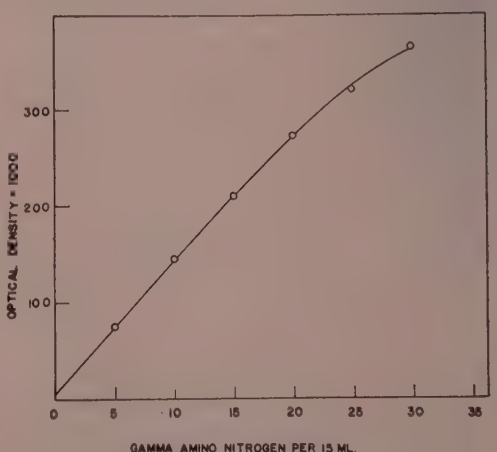

FIG. 2. Spectrophotometric determination of amino nitrogen in standard glycine-glutamic acid mixtures.

TABLE I. Recoveries of Amino Acid Nitrogen Added to Standard Uric Acid Solutions Containing Increasing Charcoal Concentrations. 125 γ amino N added to 25 ml of 3 mg % uric acid.

Charcoal added, mg	Uric acid recovered, mg	Amino N recovered, γ	%
13	.0	125	100
25	.0	125	100
44	.0	119	95
63	.0	116	92
125	.0	109	87
250	.0	98	78

TABLE II. Recoveries of Amino Acid Nitrogen When Increasing Amounts Are Added to Standard Uric Acid-Charcoal Mixtures. Amino N added to 25 ml of 3 mg % uric acid containing 13 mg charcoal.

Amino N added, γ	Uric acid recovered, mg	Amino N recovered, γ	%
50	.0	50	100
72	.0	72	100
100	.0	100	100
125	.0	125	100
147	.0	153	104
194	.0	200	103

standard ammonium chloride solutions demonstrated that boiling of the test (urine) solution at pH 9.0 to 9.5 for 25 minutes resulted in the complete removal of this substance.

The effect of activated charcoal on mixtures of uric and amino acids was studied. Bodansky reported quantitative uric acid adsorption from 430 ml of urine with 4 g of charcoal(5). In this present study with 3 mg % solution of uric acid, charcoal concentrations varying from 50 to 1,000 mg % were tested for completeness of uric acid adsorption. Table I shows that complete adsorption of uric acid occurred with all charcoal concentrations. This particular uric acid concentration was chosen since it is the highest that one would expect if urine were diluted at least 50 times as were the samples in this procedure. Non-charcoal treated urine gave amino nitrogen recoveries of about 113% of calculated. This error was in agreement with Russell.

Indiscriminate addition of charcoal resulted in low amino nitrogen recoveries. Table I shows that charcoal concentrations at and above 175 mg % resulted in losses of amino nitrogen of 5% or more. Thus, the optimal concentration of charcoal was found to be within the range of 50 to 100 mg %. It was found that this approximate quantity could be added without weighing. The amount of charcoal which covers the tip of a standard 180 mm nickel plated spatula is roughly 50 to 75 mg.

When increasing concentrations of amino nitrogen were treated with the optimal charcoal concentration, no loss of amino acids resulted (Table II). Data in Tables I and II are based on mean results of quadruplicate determinations with a precision of 1 to 2%.

Of 16 different urine specimens studied, a mean amino nitrogen recovery of 99% was obtained (Table III). The same accuracy of recovery held whether the standard glycine-glutamic acid mixture or a casein hydrolysate solution (10% amigen) was added to the urine. Analysis of the casein hydrolysate showed it to contain 740 mg % amino nitrogen with only very small daily variation in free amino content if properly stored and handled.

Since amigen contains considerable quantities of polypeptides, the hydrolysis of these substances to amino acids was a possibility. A control experiment revealed no increase in amino nitrogen when casein hydrolysate was subjected to the conditions of this determination.

When an abnormally high content of urinary protein was present, 5% sulfosalicylic acid was added to an equal volume of urine and centrifuged. The centrifugate was treated as were the other urine specimens. Recoveries of amino nitrogen from such urine yielded no loss of amino nitrogen (Table III No. 6).

Discussion. Of the several technics for determination of amino acid nitrogen, Van Slyke's titrimetric ninhydrin procedure is probably the most accurate and specific(7). However, urea is known to interfere in this procedure(8).

Of the colorimetric technics, Van Slyke notes that Folin's method as adapted by Russell is the most reliable(9). The latter author states that interference by uric acid and ammonia rendered her technic unsatisfactory for urinary determination of amino nitrogen although urea and polypeptides did not interfere.. The technic described in this paper provides simple methods for eliminating

TABLE III. Recoveries of Standard Glycine-Glutamic Acid Mixtures (S) and Amino Acids of Casein Hydrolysate (C) Added to Urine.

Urine specimen No.	Volume of urine diluted to 100 ml, ml	Amino N in urine, mg %	Amino N added to urine, γ	Amino N in urine containing added N, mg %	Recovery of added amino N, %
1	2	24.2	250 (S)	40	98
2	1.50	18.5	258 (S)	34.8	95
3	1.50	34.8	258 (S)	52.7	103
4	1.50	43	250 (S)	59.1	99
5	.50	30	270 (S)	82.5	98
6	1	12.2	245 (S)	37	101
7	1	43.3	377 (C)	79.5	98
8	1	48.3	377 (C)	87.5	102
9	1	19.5	237 (S)	83.3	100
10	1	37	313 (C)	69.3	101
11	1	38.2	313 (C)	68.3	98
12	1	16.3	285 (C)	43	96
13	2	8.8	260 (S)	21.9	100
14	1.50	11	238 (S)	26.7	100
15	1	9.5	260 (S)	35	99
16	1	27	260 (S)	52.5	100
				Mean recovery	99%

No. 1-12, casual and fasting specimens; No. 13-16, 24 hr specimens.

these complicating substances, and it is possible that the procedure might be adapted to the needs of the clinical chemical laboratory.

Conclusion. 1. Russell's colorimetric procedure for the determination of blood amino nitrogen has been adapted for urine. 2. A simple method has been described for removing the interfering substances, ammonia and uric acid, without altering the precision and accuracy of the colorimetric technic. 3. Recoveries of urinary amino nitrogen were attained with a mean value of 99%.

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Calcification. IV. Influence of Strontium and Magnesium Ions on Calcification *in vitro*.* (19193)

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It was reported earlier by one of the authors that strontium in small amounts inhibits the calcification of rachitic cartilage *in vitro* (1,2). In attempting to repeat this experiment, Marks and Shorr(3) were unable to detect such inhibition. In order to resolve this discrepancy, the subject was further investigated. As will be shown below, the results indicate the importance of magnesium ions in the inhibition of the calcifying

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TABLE I. The Influence of Sr and Mg Ions on Calcification *In Vitro*.*

Sr, mM/L	Mg, mM/L	Degree of calcification (mean)†		
		Ca × P = 40‡	Ca × P = 50‡	Ca × P = 70‡
0	0	1.5 (4+)	1.5 (4+)	2.7 (4+)
1	0		1 (4+)	
0	1		1.5 (4+)§	
.30	.70		Trace	
1.33	0			1.5 (4+)
0	1.33			2.3 (4+)
.58	.75	0		0
.58	0	1.1 (4+)		
0	.75	1.1 (4+)		

* In expressing the Ca × P product, Ca is expressed in mg % and inorganic phosphate is expressed as P in mg %. Ca was 10 mg % in the above experiments.

† The degree of calcification as evaluated in the low power microscope is as follows: 1 (4+) = complete thin line across the provisional zone; 2 (4+) = heavy line across provisional zone including primary tongues of cartilage; 3 (4+) = heavy line across provisional zone including primary and secondary tongues of cartilage; 4 (4+) = practically complete calcification of metaphysis (11).

‡ Calcifying solution, in addition to components in table, contains per liter 70 mM of NaCl, 5 mM of KCl, 22 mM of $NaHCO_3$; pH = 7.3, temperature = 36.5°, and incubation time = 18-20 hr (4 slices from 2 tibiae split among experiments; 4 to 6 rats used for each study).

§ Calcification is as extensive but not as dense as control sections.

mechanism by strontium. It is worth pointing out that a better understanding of the role of strontium in modifying the calcifying mechanism is of interest not only from the academic point of view, but also in its application in the treatment of humans. Strontium salts have been employed in an apparently contradictory manner, (a) as a means of softening bones prior to straightening them without surgical interference (4) and (b) as an adjuvant to cause more rapid mineralization of the skeleton in osteoporosis than does administration of calcium alone (5,6).

The apparent contradiction between the findings of Marks and Shorr (3) and Sobel *et al.* (2) was resolved when we employed a calcifying medium similar to that used in the experiments of Sobel *et al.* (2) which contained 0.75 millimol of magnesium ions per liter as part of the artificial serum, following the original technic of Shipley *et al.* (7). This magnesium was eliminated by many of the later investigators of *in vitro* calcification, since satisfactory calcification *in vitro* can be obtained in its absence. As seen in Table I, when strontium is used in the presence of magnesium, the inhibition of the calcifying mechanism is practically complete. In contrast, there is excellent calcification when strontium alone or magnesium alone is employed as the inhibitory ion in concentrations as high as the sum total of strontium and magnesium when both ions are employed. Thus it appears that the *intense inhibition of the calcifying mechanism by strontium observed in* vitro *calcification depends on the presence of magnesium ions.* Strontium indeed has a mild inhibitory function when tested in a calcifying solution which does not give maximal *in vitro* calcification. This is evident in Table I. The magnitude of this inhibition appears to be of the same order as that of magnesium. However, one cannot detect the inhibitory power of the same amount of strontium or that of magnesium when tested in a calcifying solution which gives more marked healing *in vitro*. This finding with magnesium is similar to that obtained by Shelling *et al.* (see Table III in ref. 8). Marks and Shorr (3) obtained excessive calcification in their control sections. Under these conditions the relatively mild inhibition due to strontium alone is difficult to detect.

These results prompted us to reinvestigate mineralization *in vitro* of bones from animals suffering from rickets due to strontium. These bones are characterized by a markedly diminished calcifiability *in vitro* (1,2), although their histological appearance is similar to that encountered in the usual type of rickets induced by a high calcium diet (9). As seen in Table II, strontium-rachitic sections placed in

TABLE II. Influence of Mg^{++} Ions on the Calcification *In Vitro* of Strontium Rachitic Bones.

Mg, in mM/L	Degree of calcification (mean)* Sr rickets	Control (Ca rickets)
0	2 (4+)	2 (4+)
1	0 to trace	2+ (4+)
	Bone sections shaken 2 hr with sol. containing 75 mM/L $CaCl_2$ prior to incubation with calcifying sol.	
0	3.5 (4+)	3.5 (4+)
1	3.5 (4+)	3.5 (4+)

* As evaluated in the low power microscope.

a calcifying medium devoid of magnesium calcify to the same extent as do the control calcium-rachitic sections. When, however, magnesium is placed in the solution, no calcification *in vitro* takes place, whereas in the same solution with the control rickets, one observes excellent calcification *in vitro*. Thus, again it appears that the *injury to the calcifying mechanism in rickets due to strontium is associated with the presence of magnesium ions* in the calcifying medium.

In other experiments, strontium-rachitic bone sections were shaken with calcium chloride in a manner previously described (10,11) for the reactivation of the calcifying mechanism, and calcified *in vitro* both in the presence and absence of magnesium. As seen in Table II, magnesium no longer had a marked injurious effect on the calcifying mechanism. From this it may be deduced that injury to the calcifying mechanism in strontium rickets is due to the interaction of strontium and magnesium ions.

The results obtained with strontium in the presence of magnesium are probably more indicative of the *in vivo* mechanism than experiments in the absence of magnesium, since magnesium is always present, both in intracellular and extracellular fluids.

These findings indicate that in the treatment of humans with strontium salts (3-6) the role of magnesium should be carefully evaluated. Moreover, these findings indicate the importance of studying the influence of various ions on calcification *in vitro*, both in the presence and absence of magnesium. Studies of the calcifying mechanism, to be published elsewhere, indicate an entirely different behavior of ions like cyanide and fluoride in the presence and absence of magnesium ions.

Summary. The intense inhibition of calcification *in vitro* by strontium ions requires the presence of magnesium ions. The injury to the calcifying mechanism in rickets due to strontium is associated with the presence of magnesium ions. The results suggest the importance of studying the influence of various ions on the calcifying mechanism, both in the presence and absence of magnesium.

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Calcification. V. Influence of Fluoride and Cyanide Ions in the Presence and Absence of Magnesium.* (19194)

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Robison and Rosenheim(1) reported that calcification of rachitic bone cartilage in inorganic medium is inhibited by 10^{-4} to 10^{-5} M fluoride, 10^{-3} M cyanide, and 10^{-3} M iodoacetate. Subsequently Gutman, Warrick, and Gutman(2) repeated and confirmed these observations in seeking to interrelate mineralization with phosphorylative glycogenolysis. Other groups of workers, however, including McLean *et al.*(3), have not obtained a significant interference by these ions that is not readily overcome by adding small increments of inorganic phosphate. More recently Marks and Shorr(4) have reported iodoacetate to be inhibitory while cyanide was found ineffectual in concentrations as high as 0.01 M.

We repeated several of these experiments and were unable at first to block calcification *in vitro* with either fluoride or cyanide. Indeed, raising the fluoride concentration to 10^{-3} M had the perverse effect of increasing, rather than decreasing, the area of mineralization. It has now been found, as shown below, that the origin of these discrepancies apparently lies in the role played by magnesium ion, whose presence is critical for the manifestation of fluoride and cyanide inhibition of calcification.

Experimental. A detailed description of the technic of *in vitro* calcification is given elsewhere(5). Bone cartilage sections were sliced from the rear tibiae of rachitic Wistar rats and suspended in the calcifying solutions from glass hooks. The four slices obtained from each animal were distributed between the experimental and control solutions. All incubations were carried out without shaking at pH 7.3 and 36.5°C. In addition to the test ions specified in the body of this paper, the calcifying solutions contained the following salts:

Salts	Molarity
NaCl*	.07
KCl*	.005
$NaHCO_3$*	.022
$CaCl_2$	.0025 (10 mg % Ca)
$NaH_2PO_4 + Na_2HPO_4$	.00161 (5 mg % P)

* The NaCl, KCl, and $NaHCO_3$ comprise the basal components of the calcifying solution.

The bone cartilage sections were stained with $AgNO_3$ to reveal the new mineral deposit and examined microscopically under 60 x magnification. The degree of calcification is designated in the following manner(6):

0 (0) —No calcification
1 (1+)—Trace
1 (2+)—Broken thin line
1 (3+)—Almost complete thin line across the provisional zone
1 (4+)—Complete thin line across the provisional zone
2 (4+)—Heavy line across the provisional zone including the primary tongues of cartilage
3 (4+)—Heavy line across the provisional zone including the primary and secondary tongues of cartilage
4 (4+)—Practically complete calcification of the metaphysis

Results. Effect of fluoride on calcification. In the absence of added Mg^{++} (*cf.* Table I) Slices of cartilage from rachitic rat tibiae were separated into two groups. The members of the control group were incubated for 18 hours in calcifying solution containing 10 mg % Ca^{++} (0.0025 M) and 5 mg % P (0.00161 M phosphate), while those of the second batch were incubated under the same conditions in calcifying solution supplemented with 10^{-4} M NaF. A comparative microscopic examination of the silver-stained tissues failed to reveal a distinct inhibition by fluoride. The mineral deposit formed in its presence appeared to be somewhat more shallow and to have a coarser honeycombed structure than the control, but the difference was not impressive. We have indicated that tibial sections in the absence of added inhibitors undergo calcification in the first 4-7 hours of incu-

* Aided by grants from the National Institute for Dental Research of the National Institutes of Health, U. S. Public Health Service, and the Damon Runyon Memorial Fund for Cancer Research.

TABLE I. Dual Effect of Fluoride in the Absence of Added Mg^{++}.

Preliminary treatment, temp 37°	Fluoride conc. in calcifying sol., moles/l	Incubation period, hr	Degree of calcification: Test tissue	Degree of calcification: Control*
	.0001	5	0 (0)–1 (2+)	1.5 (4+)
	"	18	1.5 (4+)	1.5 (4+)
	.001	5	2–2.5 (4+)†	2 (4+)
	"	18	3 (4+) †	2 (4+)
Sections suspended 2 hr in basal sol. containing 5×10^{-5} M $BeCl_2$; pH 7.3	"	18	0 (0)	2 (4+)
Sections suspended 2 hr in HCl, pH 2.7	"	18	0 (0)	2 (4+)

* Refers to degree of calcification of parallel untreated tibial slices in the absence of fluoride ion.

† Deposit has structure but lacks the sharpness and depth of the control.

bation(5). It was therefore reasoned that if fluoride does indeed retard ossification, then incubating over a period of 18 hours may obscure this effect by permitting a relief of inhibition or by permitting the reduced calcifying potential to operate for a greater length of time than is nominally required for the limited degree of calcification of the control. The incubation period was accordingly reduced to 5 hours, whereupon marked inhibition by 10^{-4} M fluoride was noted.

Although greater inhibition was anticipated at a fluoride concentration of 10^{-3} M, such was not found to be the case. Cartilage tissue incubated for 18 hours in calcifying solution containing 10^{-3} M fluoride calcified over a greater area than the control, although the deposit lacked in some degree the fine honeycombed character of the control. The difference was apparent even after 5 hours. Close inspection of the incubation flasks and test tissues failed to reveal a deposit or precipitate anywhere but in the metaphysis of the rachitic bones. *The enhanced mineralization in the presence of fluoride appears not to be an artifact but to require an active calcifying system.* Thus tissues inactivated by prior treatment with 5×10^{-5} M $BeCl_2$ or dilute HCl did not yield a deposit on subsequent incubation in fluoride-supplemented calcifying solution. The phenomenon of increased calcification at higher fluoride levels may reflect the formation of a fluorophosphate deposit(7). Composition studies would be required to establish this point.

In the presence of Mg^{++} (*cf.* Table II).

TABLE II. Fluoride Inhibition in the Presence of Added Mg^{++}.

Mg^{++} conc., moles/l	Fluoride conc., moles/l	Incubation period, hr	Degree of calcification: Test tissue	Degree of calcification: Control*
.001	.0001	5	0 (0)	2 (4+)
"	"	18	0 (0)	2 (4+)
"	.001	5	0 (0)	2 (4+)
"	"	18	0 (0)	2 (4+)
.0005	.0001	5	0 (0)	2 (4+)
"	"	18	1 (2+)–1.5 (4+)†	2 (4+)
"	.001	5	0 (0)	2 (4+)
"	"	18	1 (1+)–1.5 (3+)†	2 (4+)

* Tissues incubated in calcifying solution devoid of Mg^{++} and F^-. See Table I for effect of fluoride on calcification, and Table III for effect of Mg^{++} on calcification.

† Deposit tenuous and poorly integrated, with little depth.

While the results from the 5-hour runs underscored the initial inhibitory nature of 10^{-4} M fluoride ion, our inability to demonstrate interference with prolonged incubations was in striking contrast to the published observations(1) whose authenticity could not be doubted. A comparison of our experimental procedure with that of Robison revealed several differences, all but one of which were tentatively eliminated after some consideration. For example, the rats used in his studies were maintained on a rachitogenic diet for 3-4 weeks(8), whereas our animals were sacrificed after 2-3 weeks. Gutman and coworkers (2), however, were able to confirm Robison's fluoride experiment using rats kept on a rachitogenic diet for less than 2 weeks. This

TABLE III. Influence of Mg^{++} on the Course of Calcification.

Mg^{++} conc., moles/l	Incubation period, hr	Degree of calcification Test tissue	Control*
.0005	5	2 (4+)	2 (4+)
"	18	2.5 (4+)	2.5 (4+)
.001	5	1.5 (4+)†	2 (4+)
"	18	2-(4+)	2 (4+)
.002	5	0 (0)	2 (4+)
"	18	0 (0)	2 (4+)

* No Mg^{++} present in calcifying solution.
† Mineral deposit stains more poorly than the control, indicating greater inhibition than is apparent from the 1.5 (4+) designation.

point of difference, therefore, seemed not to be critical.

Our attention was ultimately centered on the contributory role of Mg^{++} to inhibition. Experiments were conducted in which rachitic cartilage slices were incubated in calcifying solution containing both 10^{-4} M fluoride and 1 x 10^{-3} M Mg^{++} (added as $MgSO_4$ or $MgCl_2$). Calcification failed to occur in 18 hours. Similar results were obtained when the concentration of fluoride was increased 10-fold, in marked distinction from the excessive calcification noted in the absence of added Mg^{++} (Table I). Control studies were also carried out in which the influence of Mg^{++} *per se* on the course of calcification was determined (Table III). Calcification was found to be blocked at a Mg^{++} concentration of 2 x 10^{-3} M, partially inhibited in 5 hours of incubation but not appreciably in 18 hours by 1 x 10^{-3} M Mg^{++}, and unaffected by 0.5 x 10^{-3} M Mg^{++}. These results are in essential agreement with the data of Shelling, Kramer, and Orent(9), who found no interference in 18 hours by 0.74 x 10^{-3} M Mg^{++} but complete inhibition by 1.48 x 10^{-3} M Mg^{++}.

It is clear from Tables I, II, and III and the discussion above that the degree of calcification obtained in the presence of both 10^{-3} M fluoride and 1 x 10^{-3} M Mg^{++} falls far short of the results obtained with either component alone. We also sought to determine whether small quantities of Mg^{++}, *e.g.* 0.5 x 10^{-3} M, which do not interfere with calcification, do nonetheless accentuate the inhibitory activity of fluoride ion. The data obtained (Table II) were gratifying. Strong inhibition was noted after 18 hours of incubation in calcifying solution containing 10^{-4} to 10^{-3} M fluoride and 0.5 x 10^{-3} M Mg^{++}, while complete inhibition was obtained in 5 hours, even at the 10^{-3} M fluoride level (compare with Table I).

Effect of cyanide on calcification. In agreement with Marks and Shorr(4), we were unable to detect inhibition of calcification by prolonged incubation with solutions containing 10^{-3} M KCN (*cf.* Table IV). Cartilage slices withdrawn and stained after 5 hours of treatment yielded somewhat variable results, but in general the degree of calcification relative to the control was not appreciably inhibited by 10^{-3} M cyanide. On supplementing the cyanide-containing calcifying solution with 10^{-3} M $MgSO_4$ or $MgCl_2$, however, a complete block of calcification was obtained with the shorter incubation period. Strong inhibition was observed after 18 hours.

Discussion. It is evident from the data presented above that *in the presence of magnesium,* both fluoride and cyanide ions are effective inhibitors of calcification *in vitro.* The hitherto unrecognized role of magnesium explains our previous inability to obtain inhibition with prolonged incubation in calcifying solutions containing either of the two anions but devoid of magnesium, and probably resolves the discrepancies reported by others.† While the original procedure of Shipley, Kramer, and Howland(10) called for 8 x 10^{-4} M magnesium sulfate in the artificial calcifying media, many of the later workers in the field, including the authors, have usually

TABLE IV. Mg^{++}-Dependence of Cyanide Inhibition.

Mg^{++} conc., moles/l	Cyanide conc., moles/l	Incubation period, hr	Degree of calcification Test tissue	Control*
0	.001	5	1.3(4+)	1.5(4+)
"	"	18	1.5(4+)	1.5(4+)
.001	.001	5	0(0)	2(4+)
"	"	18	1(1+)-1(3+)†	2(4+)

* Degree of calcification in the absence of added Mg^{++} and CN^-. See Table III for the effect of Mg^{++}.
† Subsurface deposit.

† The reader is referred to pp. 17 and 20 of reference 12 and pp. 199-200 of reference 4.

omitted magnesium, since calcification proceeds readily in its absence. This is clearly the case with Marks and Shorr(4), whose failure to detect cyanide inhibition may be attributed to the lack of magnesium in their incubation solution. On the other hand Robison and Rosenheim(1), and later Gutman, Warrick, and Gutman(2), did observe interference by cyanide and fluoride. But they apparently did not associate the inhibition phenomenon with the specific activity of magnesium, which was included in their solutions in physiological concentration (0.001 M).

While fluoride ion is a potent inhibitor of calcification in the presence of an adequate concentration of magnesium, it appears to enhance calcification *in vitro* when used at a level of 10^{-3} M in the absence of added magnesium. This phenomenon suggests that in the early stages of bone, and possibly tooth development, the incorporation of fluorine in the apatite lattice(7) may proceed best in the presence of minimal amounts of magnesium. A comprehensive study of the relationship between dietary and serum magnesium and the effect of fluorine on the *early* growth of teeth seems to be called for.

The known presence of small amounts of magnesium in the experimental bone cartilage slices probably accounts for the initial inhibitory action of 10^{-4} M fluoride in calcifying solutions devoid of magnesium. On more prolonged incubation, much of this magnesium diffuses out of the cartilage(11). The resultant decrease in cartilage magnesium may then account for the observed relief of inhibition.

Presumptive evidence for the action of fluorine as an enzyme poison is afforded by its block of calcification in concentrations which are too low to affect the solubility of bone salts. Although a complete spectrum of the enzyme content of cartilage is not available, the ostensible requirement of magnesium for the manifestation of fluoride inhibition of calcification may be tentatively correlated with the presence of enolase in rachitic rat tibiae(12). From their studies on the properties of crystalline enolase, Warburg and Christian(13) have concluded that its specific inhibition by fluoride involves the formation of an inactive protein—magnesium fluorophosphate complex. It is significant that the activity of the enzyme, at a given concentration of fluoride ion, decreases with increasing concentration of magnesium. This may explain the need for adding magnesium in order to effect a decisive fluoride block of calcification. Further investigation is under way to determine whether the parallelism between the inhibitory action of magnesium and fluoride ions on enolase activity and on calcification is fortuitous.

The mechanism of cyanide inhibition is less readily amenable to interpretation(12). The deficiency in cartilage of cytochrome oxidase (11,14,15) and of respiratory and oxidative enzymes in general(11,14,16) seems to preclude the specific activity of cyanide here as an oxidative poison. Work in progress may help to elucidate the role of magnesium as a mediator of the cyanide inhibition of calcification.

Summary. 1. The *initial* rate of calcification of rachitic cartilage sections in inorganic media is markedly reduced in the presence of 10^{-4} M fluoride ion. With more prolonged incubation there is a relief of inhibition unless magnesium is included in the calcifying solution. 10^{-3} M fluoride is similarly effective in preventing mineralization provided the solution contains an adequate amount of magnesium; in its absence, the initial interference by 10^{-3} M fluoride is rapidly obscured by a secondary effect, interpreted as incorporation of fluoride in the growing mineral deposit, which gives rise to an apparent increase in calcification over the control. 2. Cyanide ion blocks calcification in the presence, but not in the absence of magnesium.

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Non-Effect of Cortisone on Growing Bones of Mice, Guinea Pigs and Rabbits. (19195)

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When cortisone acetate is administered to growing rats, a zone of increased density appears at the ends of the long bones and at the costochondral junctions(1). Such a metaphyseal area of increased density is composed of spicules of calcified cartilagenous matrix encased in bone. The pathogenesis of this change has been interpreted to be due to a decrease in normal osteolytic sequences(1). It seemed appropriate, therefore, to examine the skeletal tissues of several other species to which cortisone acetate* had been administered in order to determine whether changes similar to those encountered in the rat might be observed.

Experimental Mice. Weanling and full grown mice were given cortisone acetate in daily doses ranging from 10 to 75 mg per kilo. Animals were sacrificed from the 3rd up to the 23rd day. X-rays were taken of the upper ends of the tibiae; sections of these bones were studied microscopically. Aside from a retardation of growth in the young animals receiving 50 and 75 mg cortisone per kilo per day no changes were found in the metaphyses.

Guinea pigs. Guinea pigs weighing 180 to 200 g at the beginning of the experiment were given 5, 12.5, 25 and 50 mg cortisone acetate per kilo per day; animals were sacrificed at intervals from the 12th to the 37th day of treatment. X-rays and microscopic sections of the ribs and upper ends of the tibiae showed no changes save a retardation of growth in the animals receiving 50 mg cortisone per day.

Rabbits. Young rabbits, weighing 800 to 1000 g were given 5, 10, 25, 40, and 80 mg cortisone acetate per kilo per day and were examined at intervals after the 12th to the 37th day of such treatment. X-rays and sections of the ribs showed no changes save retardation in growth in the animals receiving 80 mg cortisone per day.

Discussion. Changes which may be produced in the rat's skeleton when appropriate doses of cortisone acetate (40-50 mg per kilo per day) are administered have been interpreted as being due to a failure of normal resorptive processes(1). In this they resemble bony alterations which may be encountered in the same species as a result of the influence of large amounts of estrogen. Under such circumstances normal osteolytic sequences appear to be inhibited(2,3). Since, in contrast to the rat, the effects of estrogen on the skeletal tissues of the mouse are so different and so striking, *i.e.* a tremendous stimulation of endosteal activity(4), a study of this species was approached with great interest. The results of cortisone administration, however,

* The cortisone acetate (Cortone) was kindly supplied by Merck and Co., Rahway, N. J.

were entirely negative. Any specific response of the guinea pig or rabbit to cortisone was also lacking. The effect of cortisone on the rat would, therefore, appear to be unique among the species so far studied. In this respect, it should be recalled that, aside from its action on the mouse which was referred to above, estrogen appears to have no specific effects on all other mammalian species which have been studied so far save the rat(3).

Summary. Varying amounts of cortisone acetate were administered to growing mice, guinea pigs, and rabbits. This hormone in the doses used appears not to have an effect such as has been observed in the rat on the skeletal tissues of these species. Since large amounts led to a retardation in growth, it would seem unlikely that the mouse, guinea pig, or rabbit is less sensitive to the hormone.

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An Improved Recording Rotameter. (19196)

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In many physiological studies it is desirable to obtain continuous measurement and registration of the mean rate of blood flow through a particular blood vessel. An optically recording rotameter which was designed for this purpose(1,2) operated on an electromagnetic principle, and changes in blood flow through the rotameter were detected and translated into proportionate deflections of a light beam reflected from a galvanometer mirror. The use of an electronic circuit involving an oscillator, amplifier, and rectifier made the apparatus relatively difficult to construct and maintain without some degree of technical knowledge. For this reason an attempt was made to simplify and improve the flow metering device without sacrificing sensitivity or accuracy. The construction and performance of the redesigned instrument is reported here.

In Fig. 1 is shown, in sectional view, the metering and detecting portions of the rotameter proper. Construction details are given in the legend. The metering chamber illustrated is designed to cover a flow range of 0-400 cc/min. Other metering chambers which are interchangeably attachable to the detecting portion have been constructed for individual ranges having top flows of 100, 200, 800, 1600, and 3000 cc/min.

The electrical circuit diagrammed in Fig. 2 was constructed from standard parts and assembled in a small control box measuring 3 x 4 x 5 inches.

The entire apparatus is shown in Fig. 3. By viewing the 0-25 microammeter (100 arbitrary scale divisions) the changes in flow through the rotameter could be noted visually. A simple optically recording galvanometer was constructed as follows. The jewel bearings of a 0-200 microammeter were removed, the coil suspended by two nylon filaments and a 3 mm x 3 mm mirror cemented to the counterbalance end of the pointer arm. A rectangular opening was cut in the meter case opposite the mirror and a glass window sealed in place with wax. To provide a stable support, pointed legs were attached to the case through the three mounting holes. A variety of commercially available mirror galvanometers may also be used for recording on sensitized paper or film. The maximum output is approximately 25 μA and 0.1 volt. With supplementary DC amplification, satisfactory records have been made with instruments employing a hot stylus or a pen writer.

Calibrations of the instrument remain con-

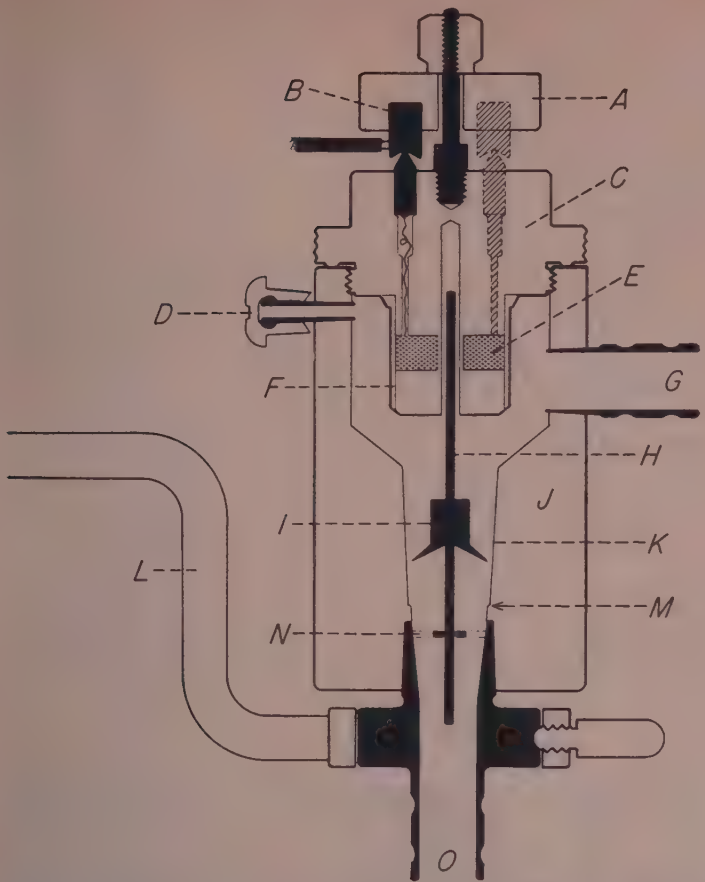

FIG. 1. Full-scale sectional view of 0-400 cc/min rotameter constructed of Lucite or Plexiglass with silver plated brass fittings except where otherwise noted. A: contact holder; B: one of 3 contacts communicating with the 2 terminals and common connection of detecting coil and balancing (non-inductive) coil; C: detecting assembly; D: rubber cap through which air may be removed during filling; E: detecting and balancing coils sealed in wax; F: protecting sleeve; G: outflow spout; H: annealed soft iron silver plated float wire, .051 inch diameter; I: brass float disc. Float is shown in position it would occupy in middle of flow range. Float is 15/32 inch above float rest when at top of flow range. J: detachable metering chamber; K: metering portion, bored with .080 inch per inch taper; L: rotameter support; M: float rest at zero flow; N: float guide; O: inflow spout.

stant within less than 5% variation over 6-8 hours continuous running. Variations in the calibration due to line voltage fluctuations are eliminated by using a constant voltage transformer to supply current to the set. The calibration plots (Fig. 4) are only slightly curved or sigmoid in shape due to the fact that the dimensions and position of the detecting coil and the float wire are adjusted so that the maximum rate of change in reactance occurs in the middle of the flow range. Because of this feature the bridge is not balanced at the lowest float position (zero flow) since the float wire occupies a position partly within the coil. The positive reading on the meter (about ⅛ full scale) is nullified by turning the zero adjusting screw until the meter reads zero.

Calibration of the rotameter is not greatly affected by changes in viscosity of the blood (Fig. 4). The curves obtained with fluids having different viscosities were not significantly different except at the lower end of the

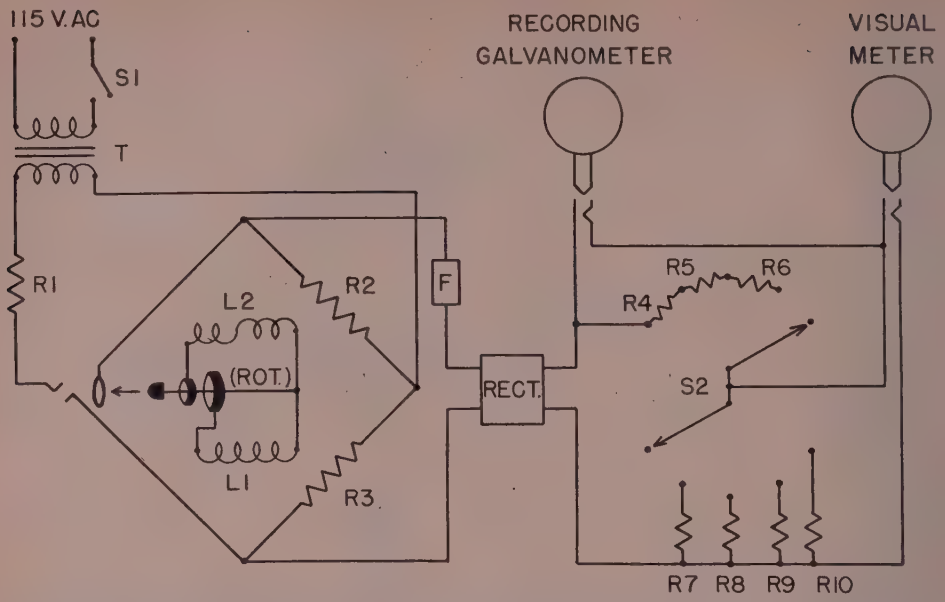

FIG. 2. Wiring diagram of electrical circuit. F: 1/100 amp. fuse; L1: 1600 turns No. 40 enameled copper wire, 110 ohms resistance; L2: 110 ohm, non-inductively wound coil. Resistors: R1, 120-180 ohms, 2 watt; R2 and R3, 400 ohms, Manganin or Advance wire wound, 5 watt; R4, 100 ohms; R5, 330; R6, 750; R7, 20K; R8, 10K; R9, 6800; R10, 5600. Rect.: type M Conant full wave rectifier; Rot.: rotameter detecting assembly with jack; S1: off-on switch; S2: recording galvanometer sensitivity selector switch; T: chime transformer (A.P. Foster), 13V secondary; recording galvanometer, 0-200 μA meter with mirror mounted on hand; visual meter, DC, 0-25 μA meter.

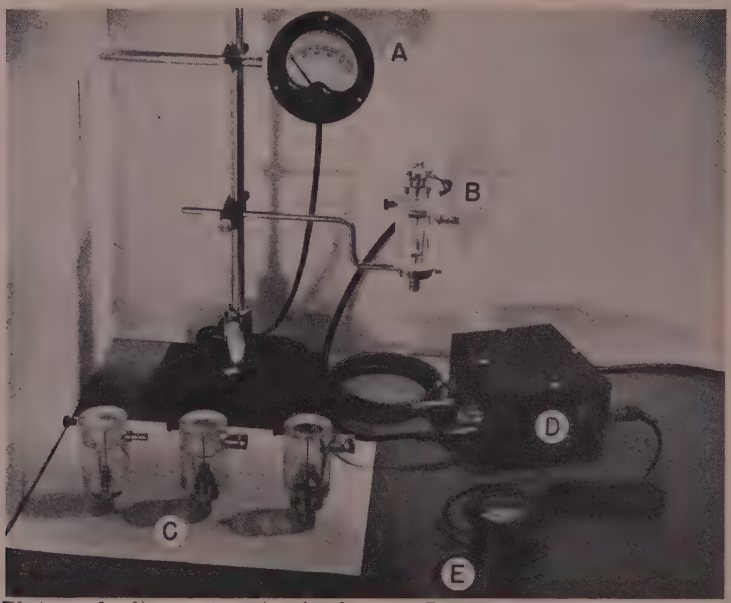

FIG. 3. Photograph of apparatus. A: visual meter; B: rotameter; C: interchangeable metering chambers accommodating different flow ranges; D: control box; E: recording mirror galvanometer.

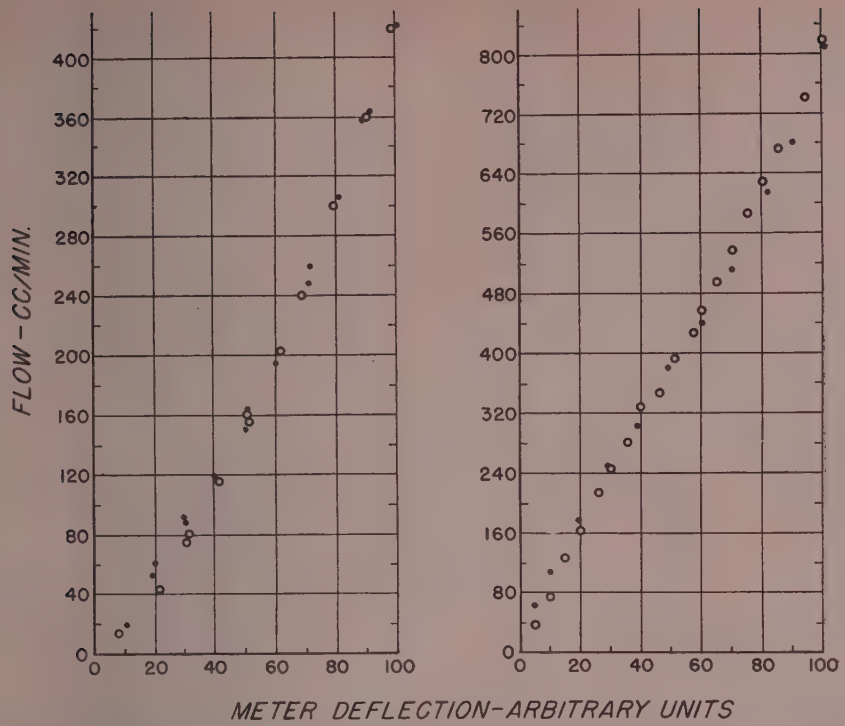

FIG. 4. Calibration curves obtained with fluids having different viscosities. *Left*: metering chamber with range of 0-400 cc/min; dots: calibration points using methyl cellulose solution, specific viscosity 3; open circles: specific viscosity 6.2. *Right*: metering chamber with range of 0-800 cc/min; dots: calibration points using water, specific viscosity 1; open circles: methyl cellulose solution, specific viscosity 4.6.

flow range. The shape of the float disc was copied in part from a design* which was found to eliminate viscosity effects in commercial size rotameters.

Pulsatile flows may be measured accurately within 5%, provided the float does not touch the float rest at its lowest position and its excursion does not exceed the upper limits of the metering area. There is no mechanical dampening of the motion of the float. If the flow pulse is very large, and excessive oscillation of the meter and galvanometer make it difficult to estimate the mean rate of flow, a pulse dampening chamber partly filled with air may be interposed between the source of blood supply and the rotameter.

To minimize pressure drop across the instrument the metering chamber and fittings were made with a large internal diameter, and the float was kept as light as possible for each metering unit. The pressure drop across the rotameter and fittings was measured with each of six metering chambers having different flow ranges. A relatively small pressure head (2-3.5 cm of water) was required to operate the instrument at all rates of flow (see Fig. 5). By increasing the bore of the metering chamber and the diameter of the float the pressure drop could be reduced even further. However, a proportionately larger volume of blood was required to fill the metering chamber, and, in addition, the velocity of the flow through the instrument was reduced to a level where settling of blood cells could occur at low rates of flow.

Since the pressure drop through the entire circuit determines the extent to which flow will be hindered, it is particularly important

* "Ultra-Stabl-Vis," Fischer and Porter Co., Hatboro, Pa.

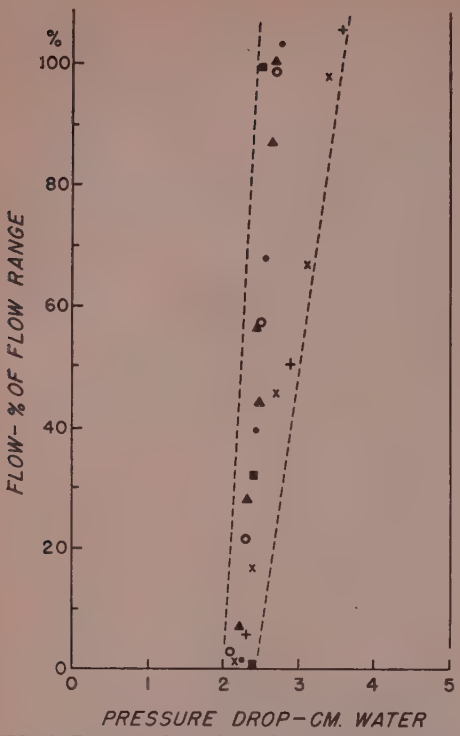

FIG. 5. Pressure drop through rotameter with different metering chambers and at different rates of flow. Ordinate: flow is expressed in terms of percentage of the nominal capacity of each metering chamber. Dots: metering chamber with nominal capacity of 100 cc/min; open circles: 200 cc/min; squares: 400 cc/min; triangles: 800 cc/min; plus signs: 1600 cc/min; ×'s: 3 liters/min.

that the connecting tubing and cannulas used in association with the rotameter be of maximum bore and minimum length.

In different acute experiments and under a variety of conditions, satisfactory continuous records have been made of the rate of blood flow through carotid, renal, femoral, and coronary arteries and through jugular, venacaval, renal, femoral, and coronary sinus veins. Illustrations of typical records may be found in other reports(3-6).

Summary. (1) A simplified and improved flow rate meter is described. The instrument is basically a rotameter, the float of which is vertically displaced in proportion to the rate of flow and its position detected electromagnetically. The rate of flow is indicated on a microammeter and a recording galvanometer. The metering chamber and float are designed so that viscosity effects are minimized and the pressure drop through the instrument reduced to less than 3.5 cm of water at any rate of flow. (2) To obtain maximum sensitivity over a given flow range, different metering chambers, with capacities ranging from 100 cc to 3 liters per minute, can be used interchangeably with one detecting unit.

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Prevention of DL-Methionine Toxicity in Rats by Vitamins E, B_{12}, Folacin, Glycine and Arginine.* (19197)

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The toxic effect of high levels of DL-methionine was noted by Earle, Small, and Victor

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(1). Brown and Allison(2) showed that 1.7% dietary arginine partially prevented loss in weight of rats on a diet containing 12% casein and 4.8% DL-methionine. They also noted an increase in creatinine excretion of rats on the high methionine diet. Van Pilsum and Berg(3) and Cohn and Berg(4) recently reported that the toxic effects of excess dietary (1.8%) DL-methionine in rats were counteracted by the addition of molecular equivalent quantities of glycine and arginine to the diet, while guanidoacetic acid was ineffective. Their diet contained ample quantities of vit. E, folacin, and vit. B_{12}.

It has been shown(5) that methionine protects against the acute toxicity of carbon tetrachloride under special dietary conditions. Vit. E and vit. B_{12} share this property(6). Hove(7) noted the similarity of the symptoms of vit. E deficiency and carbon tetrachloride poisoning in rats. Data at this laboratory (8) show that vit. E increases the utilization of methionine in methylating guanidoacetic acid to creatine *in vitro*. Hove and Hardin (6) found that vit. E and folacin stimulated the growth of young rats on a 10% protein diet; therefore, it became of interest to determine if vit. E, folacin, and vit. B_{12} influenced the toxicity of excess methionine.

Experimental. The basal diet contained 10% casein purified as reported by Salmon (9), 9% lard, 1% cod liver oil, 4% salt mixture(9), 0.5% DL-methionine, and sucrose to 100%. The basal diet contained the following levels of pure vitamins per g: thiamine, riboflavin, and pyridoxine, 5 μg each; calcium pantothenate, 25 μg; niacin, 40 μg; choline chloride, 2 mg; i-inositol, 0.2 mg; and 2-methyl 1-4 naphthoquinone, 2 μg. The supplements used were added to the basal diet as follows: α-tocopherol acetate, 0.01%; crystalline vit. B_{12}, 3 μg %; and folacin, 0.1 mg %. In addition to the methionine furnished by the casein (about 0.3%), the diet was supplemented with 2.5% DL-methionine or 2% above the level in the basal diet. Glycine (1.0%) and L (+) arginine (2.3%) were added in some cases. These were molecularly equivalent to 2% methionine. Weanling albino rats of Sprague-Dawley origin weighing 45 $\pm$ 5 g were fed the basal diet until average weight was 65 $\pm$ 5 g. At that time the rats were distributed among the dietary treatments according to sex and litters. A total of four experiments were conducted with 3 to 4 rats per dietary treatment. The rats were housed individually in metal cages and food and water were provided *ad libitum*. The animals were weighed at the beginning of the experiment and weekly thereafter. At the end of 4 weeks, urinary creatine analyses were made by the method of Eggleton, Elsden and Gough (10); 2 ml of carbon tetrachloride per kg body weight were then injected and urinary creatine was again determined on the rats surviving 24 hours. The number of deaths that occurred during the first 48 hours following injection of carbon tetrachloride were recorded.

Results. When the DL-methionine was increased by 2 percentage points in the basal diet, growth rate of the rats for the 4-week period was decreased from 61 g to 22 g, or a decrease of 64% (Table I). When α-tocopherol acetate, folacin, and vit. B_{12} were added to the high methionine diet, the growth inhibition due to toxicity of the methionine was 39%. A statistical analysis of the data by means of Fisher's t test indicated that this was a highly significant protective effect. A t value of 4.7 was obtained on comparing the high methionine groups with and without the combined vitamin supplements.

When the vitamins were added to the high methionine diet either individually or in pairs, it was noted that the folacin-supplemented diet gave slight, but significant protection against methionine toxicity (t value 2.9). However, when α-tocopherol acetate was combined with folacin, very significant partial protection was obtained (t value 9.0). α-Tocopherol acetate and vit. B_{12}, alone or in combination, were without effect on growth.

The addition of molecular equivalent quantities of glycine and arginine gave a very significant partial protection (t value 7.0) against the high methionine, which was approximately the same as that obtained by the vitamin supplements. When supplements of tocopherol acetate, folacin, and vit. B_{12} were

TABLE I. Influence of Dietary Supplements on Toxicity of DL-Methionine, Creatine Excretion and Sensitivity to Carbon Tetrachloride in Rats.

Addition to 10% casein diet	No. rats	4 wk gain		Urine creatine (mg/day/100 g body wt)		Survivals 48 hr after 2 ml CCl_4/kg body wt	
		g	S.E. ±	Without CCl_4	With CCl_4*	No.	%
DL-meth. (.5%)	14	61	4.7	1.5	5.4	6	43
" + vit. E, B_{12}, folacin	14	69	2.8	.7	2.3	14	100
DL-meth. (2.5%)	15	22	1.5	.8	11.0	3	20
" + vit. E, B_{12}, folacin	15	37	2.8	.5	4.3	8	53
" + vit. E	11	22	.7	.3	.3	6	55
" + vit. B_{12}	11	22	1.3	.2	0	5	45
" + folacin	11	27	.9	.1	.4	4	36
" + vit. B_{12} and folacin	11	25	.6	.2	.4	5	45
" + vit. E and B_{12}	11	23	1.3	.2	—	2	18
" + vit. E and folacin	7	38	1.3	.2	—	1	14
" + gly. and arg.	4	43	2.6	2.7	17.2	1	25
" + gly. and arg. + vit. E, B_{12}, folacin	4	66	5.9	4.6	3.8	4	100
" + guanidoacetic acid	4	16	5.6	31.0	28.9	1	25
" + guanidoacetic acid + vit. E, B_{12}, and folacin	4	32	3.2	28.1	15.3	3	75

* Urine collected for 24 hr after the CCl_4 injection.

supplied in combination with the glycine and arginine, the growth inhibition due to the excess methionine was eliminated, which confirms the work of Cohn and Berg(4). Guanidoacetic acid did not protect against methionine toxicity. The addition of the 3 vitamins along with the guanidoacetic acid produced significantly higher growth than the guanidoacetic acid alone, (t value 6.3).

The urinary creatine of the rats receiving high methionine was decreased to about half the value of the urinary creatine of rats on the basal diet. The urinary creatine of the rats receiving glycine and arginine was increased above the level in the control rats. The addition of α-tocopherol acetate, folacin, and vit. B_{12} increased the urinary creatine even more. The addition of guanidoacetic acid to the basal diet produced a very high excretion of apparent creatine. Of this apparent creatine, about half could have been due to the excretion of guanidoacetic acid, since the method used for creatine analysis will give a color with guanidoacetic acid equivalent to 1/10 that produced with creatine. The high methionine diet afforded less protection against creatinuria caused by carbon tetrachloride than did the basal diet. In all cases, the vitamins reduced the creatinuria due to CCl_4.

The percentage of rats surviving in each group at the end of 48 hours after injections of 2 ml carbon tetrachloride per kg body weight is also shown in Table I. The addition of the 3 vitamins to the basal diet afforded 100% protection against this level of carbon tetrachloride. Rats on the high methionine diet were more sensitive to carbon tetrachloride than were rats on the basal diet except when the diet was supplemented with glycine, arginine, and the combined vitamins. Vit. E, folacin, or vit. B_{12} seem to have comparable protective effects against the toxicity of carbon tetrachloride given to the rats on the high methionine diet. Combinations of vit. E with vit. B_{12} or folacin were without effect.

Since glycine was involved in protecting rats against methionine toxicity, the reverse was studied. The data in Table II show that either α-tocopherol acetate (.01%) or DL-methionine (1.0%) prevented death due to toxicity of 4% dietary glycine. Partial protection against the growth depression due to this level of glycine was provided by either supplement, while the combination of vit. E and methionine gave complete protection.

Discussion. There may be several explanations of why glycine and arginine, in the presence of the 3 vitamins, protect against excess DL-methionine.

TABLE II. Glycine Toxicity in Rats Counteracted by Vitamin E and Methionine. 4 to 6 rats per group.

Additions to 10% casein diet	4 wk weight gain with % dietary glycine at 0	4	Depression of growth rate due to glycine (%)
None	36 g	24 g*	33
Vit. E, .01%	45	34	24
DL-methionine, 1%	64	49	23
Vit. E and methionine	69	63	8

* Five of the 6 rats in this group died during the 3rd and 4th wk. The value given is the 3-wk gain extrapolated to 4 wk.

An hypothesis advanced by Cohn and Berg (4) is that the detoxification of excess methionine is accomplished by supplying the proper substances to utilize the methyl groups. Data from this laboratory(5) show that α-tocopherol acetate increases the utilization of methionine in the synthesis of creatine *in vitro* from guanidoacetic acid. The growth data show that supplementary vit. B_{12} or vit. E, alone or together, are not effective in detoxifying methionine. It can be reasoned that, since the precursors of creatine, *i.e.*, glycine and arginine, in the presence of the combined vitamins are very effective in detoxifying methionine, the detoxification is accomplished by using up excess methyl groups to form creatine. The urinary creatine was increased when glycine and arginine were fed. It was increased still more when vit. E, folacin, and vit. B_{12} were added along with the glycine and arginine. This increase was approximately proportional to the increase in body weight caused by the addition of these factors.

An alternate explanation could be that the high methionine creates an amino acid imbalance and precipitates glycine and arginine deficiencies in the animal.

Guanidoacetic acid may not take the place of the glycine and arginine in detoxifying the methionine because these specific amino acids have become dietary essentials. It could be that, for the synthesis of creatine, the reaction of the guanidyl group from arginine with the acetate and methyl groups from glycine and methionine, respectively, is a coupled reaction and will take place more readily when all of these are present simultaneously.

Summary. (1) The toxicity of DL-methionine, fed to rats at a 2% dietary excess level, was more pronounced in the absence of dietary vit. E, folacin, and vit. B_{12}. The combination of vit. E and folacin was as effective for growth as all 3 vitamins together. Without the 3 vitamins, growth in 4 weeks was 32% of the normal, as compared with 54% of normal upon their addition to the diet. Supplements of glycine and arginine in the absence of these vitamins produced growth that was 62% of normal. (2) The methionine toxicity was prevented by molar equivalent supplement levels of glycine plus arginine if the 3 vitamins were added to the diet (95% of normal), but was not prevented in the absence of these factors. (3) Urinary creatine and sensitivity to acute carbon tetrachloride were determined for all rats. (4) Complete protection against glycine toxicity was given by dietary methionine with vit. E.

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Plasma Antihemophilic Activity Following Total Body Irradiation.*† (19198)

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There is no general agreement at present regarding the nature of the coagulation defect which develops following total body irradiation. No deficiency of fibrinogen exists(1); instead, fibrinogen levels are often elevated. Likewise there is no deficiency of Ac-globulin (2) or of prothrombin(1,2). The severe thrombocytopenia appears to be of major importance in the delayed clotting, although many workers have denied that the low platelet level is the sole basis of the coagulation defect. It has been suggested that either an excess of a heparin-like circulating anticoagulant(1) or a deficiency of plasma thromboplastin(3) may contribute to the clotting abnormality. Recently it has been shown in irradiated dogs that thrombin is formed slowly during clotting(2). One component required for prompt evolution of thrombin in clotting blood is the antihemophilic factor (AHF). With the recent development of an assay procedure for AHF(4), it became possible to determine if alterations in this clotting factor occur after whole body irradiation.

Procedures. Ten healthy mongrel dogs were given single doses of total body X-irradiation (600 r, 2000 kv) by a procedure described previously(5). Platelet and leucocyte counts and hematocrit values were determined at frequent intervals on all dogs. For platelet counts the method of Brecher and Cronkite (6) was used on five of the dogs, and a modification of Nygaard's method(7) on the other animals. Two measures of the clotting defect were used: (a) clotting times of venous blood, using either a modified Lee-White procedure (two tubes, 10 x 75 mm, 28°C) or the procedure of Jackson *et al.*(2) (three tubes, 12 x 75 mm, 37°C) and (b) the rate of loss of prothrombin from clotting blood, as determined by the prothrombin utilization test(8). Prothrombin determinations were made by a modified two-stage procedure(4). AHF assays were performed by a method recently described(4).

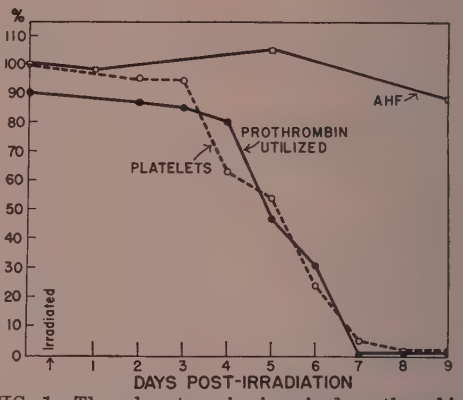

FIG. 1. Thrombocytopenia, impaired prothrombin utilization and stable plasma AHF following irradiation of 19.5 kg ♂ dog. Only the 1 hr prothrombin data are plotted. Control platelet count 614,000/mm³, Nygaard method.

Results and discussion. All the dogs developed a severe irradiation reaction and died within 7-15 days. In all animals at the height of the reaction, severe anemia, leucopenia and thrombocytopenia (0-12,000 per mm³) were present. Impaired clotting was observed. The clotting time by the Lee-White procedure ranged from 12-40 min (normal controls, 6-8 min) and by the Jackson procedure ranged from 70-300 min (normal controls, 10-20 min). In clotting blood, prothrombin utilization was impaired; less than 10% was consumed in one hour. The response of one animal, typical of this group of dogs, is shown in

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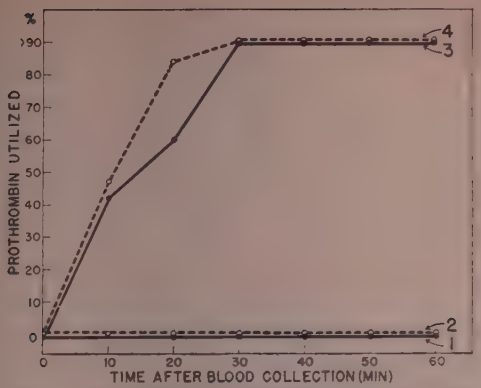

FIG. 2. Transfusions of hemophilic dog with plasmas from irradiated and normal dogs. Recipient, ♀, 21.6 kg; dose 2 ml/kg. See text for explanation of curves.

Fig. 1. Beginning on the fourth day after irradiation, a progressively increasing thrombocytopenia developed. Concomitantly, there was impaired utilization of prothrombin in clotting blood. Plasma AHF levels, however, remained within normal limits. This dog died on the 9th day following massive hemorrhage into the intestine. Autopsy studies revealed in addition widespread destruction of lymphoid tissue similar to that observed by Tullis(9) in swine used in the Bikini atomic bomb tests.

Plasma AHF assays were done on 9 of the 10 dogs at the time the clotting defect was most pronounced (7-13 days after irradiation). The mean value obtained was 97.5% of normal plasma; 95% confidence limits were 81.6-113.4. This value for AHF is comparable to that observed in a series of normal dogs(4).

Further tests of antihemophilic activity of the irradiated dogs were made by transfusing their plasmas into hemophilic dogs and comparing the changes in the hemophilic clotting defect with those produced by normal plasma transfusions. The results of one of 3 experiments of this type are shown in Fig. 2. In this experiment whole blood obtained from a dog 12 days after total body irradiation showed no detectable utilization of prothrombin in one hour (Curve 1, Fig. 2). Citrated plasma, prepared from this dog at the same time by the silicone technic(8), was transfused promptly into a hemophilic dog. Like the irradiated dog, the hemophilic recipient prior to transfusion showed no prothrombin utilization (Curve 2, Fig. 2). Immediately after transfusion, however, the prothrombin in the hemophilic blood disappeared rapidly (Curve 3, Fig. 2). This corrective effect was similar to that produced by a transfusion of citrated normal plasma (Curve 4, Fig. 2), given to the same hemophilic animal after the beneficial effects of the original transfusion had disappeared.

Previous work has indicated that formed elements, particularly platelets, are necessary for AHF to manifest itself(10). In the above experiments, plasma AHF was active in the presence of the full complement of platelets furnished by the hemophilic blood. In the following experiment, a study was made of the clotting rate in the mixtures of irradiated dog's blood with either hemophilic platelet-poor plasma or whole blood. With platelet-poor hemophilic plasma (Series 1, Table I), it will be observed that rapid conversion occurred when platelets were abundant in the irradiated dog's blood. When thrombocytopenia developed, however, prothrombin utilization was retarded. On the other hand, the addition of the irradiated dog's blood to whole hemophilic blood resulted in prompt utilization of prothrombin throughout the post-irradiation period (Series 2, Table I). A similar mutually corrective effect of blood from patients with idiopathic thrombocytopenia and with hemophilia has been observed previously(11). It would appear that the blood from irradiated dogs is potentially capable of clotting at a normal rate, provided that an adequate number of platelets is available.

In view of evidence that certain globulins are produced by lymphoid tissue(12), it is of interest that AHF remained at normal levels in these experiments despite severe damage to the lymphoid system. Previous data indicated that AHF, unlike some other proteins of the clotting reaction, is not reduced in severe liver damage(4). Apparently neither an intact lymphoid system nor an intact liver is needed for the maintenance of the antihemophilic activity of plasma.

Summary. 1. Plasma antihemophilic ac-

TABLE I. Effect of Dog Blood Collected Daily Following Irradiation on Platelet-Poor Hemophilic Plasma and Whole Hemophilic Blood.

Days after irradiation	Test series 1 .5 ml irradiated dog whole blood* .7 ml platelet-poor hemophilic plasma†		Test series 2 .5 ml irradiated dog whole blood* 1.2 ml hemophilic whole blood*‡	
	% prothrombin utilized in 1 hr	No. platelets per mm³ mixture	% prothrombin utilized in 1 hr	No. platelets per mm³ mixture
2	90	243000	90	432000
3	90	240000	82	404000
4	85	161000	90	356000
5	81	138000	90	308000
6	79	61300	90	302000
7	17	12750	90	204000
8	28	2300	90	198000
9	18	1275	90	218000

* Whole blood freshly drawn, no anticoagulant, immediate mixing. Irradiated dog same as in Fig. 1.

† Prepared by centrifugation at 5°C at about 14000 g for 2 hr, silicone technic, no anticoagulant.

‡ Equivalent to .7 ml plasma.

tivity of dogs subjected to total body irradiation remained at normal levels. 2. Whole hemophilic blood with a full complement of platelets accelerated the clotting of irradiated dog's blood, while platelet-poor hemophilic plasma did not.

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Studies on Purine and Pyrimidine Requirements of a Strain of *Streptococcus faecalis*. (19199)

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In an earlier paper(1) it was reported that a strain of *Streptococcus faecalis*, subsequently designated *S. faecalis* AB, required uridine or other pyrimidine nucleosides and nucleotides for optimum early growth. In that study, the authors used a basal medium containing free purine and pyrimidine bases to obtain maximum growth response to uridine. Snell *et al.*(2-4) reported that purine and pyrimidine bases had no stimulatory effect on the growth of *S. faecalis*, while Barton-Wright (5) and Luckey *et al.*(6), without discussion

of specific requirements, included these compounds in a basal medium for microbiological assays with this organism.

In order to determine whether the inclusion of uridine in the medium modified the utilization of other pyrimidines or purines by *S. faecalis* AB, a more detailed study of purine and pyrimidine requirements was undertaken.

Experimental. The materials and methods used are similar to those described previously (1) except that all purines and pyrimidines were omitted from the basal medium. Individual nucleic acid derivatives* were added to the culture tubes separately and with uridine in order to compare growth responses in each case. Supplements were assayed at five levels ranging between 0.7×10^{-5} M and 7.4×10^{-5} M. Since no inhibition of growth was noted with more than the optimum quantity of supplement in the basal medium, the concentration selected for the presentation of comparative data is that (3.7×10^{-5} M) which produced the maximum response with the least active compound. In a preliminary analysis and purification by methods of Cohn(7) and Carter(8), it was found that only guanine and guanylic acid showed a difference in microbiological response between the commercial and purified preparations. Therefore, with the exception of guanine and guanylic acid, commercial products were used in subsequent experiments without further purification. Growth in triplicate tubes was determined turbidimetrically and the average reading recorded at incubation times which showed maximum differences in growth rates, usually 16 and 24 hours at 30°C.

Results. The data obtained from the addition of individual purines and pyrimidines to the synthetic basal medium are shown in Table I. Of the compounds tested, none of the purine bases or their ribosides and ribotides gave a significant increase in growth as compared to the basal medium. Of the pyrimidine bases tested, only orotic acid stimulated growth while all of the pyrimidine ribosides and ribotides stimulated growth above that obtained with the basal medium.

TABLE I. Comparative Growth Responses of *S. faecalis* AB to Purines, Pyrimidines and Related Compounds. 23 hr incubation at 30°C.

Supplement (3.7×10^{-5} molar)	Growth: optical density × 100
None	11.5
Adenine	11.4
Guanine	12.1
Adenine + guanine	13.1
Hypoxanthine	10.3
Thymine	14
Cytosine	12.6
Orotic acid	21
Uracil	13.2
Uracil + adenine + guanine	12.9
Uracil + xanthine + cytosine	12
Xanthine	12.1
Adenosine	11.1
Guanosine	13.8
Uridine	21.4
Cytidine	19.8
Thymidine	13.5
Adenylic acid	11.2
Guanylic acid	12.2
Uridylic acid	22
Cytidylic acid	21.2

TABLE II. Growth Response of *S. faecalis* AB to Combinations of Purine and Pyrimidine in Presence of Uridine. Incubation at 30°C.

Supplement* (3.7×10^{-5} molar)	Growth: optical density × 100 16 hr	24 hr
None	1.2	12.2
Uridine	5.2	37.4
" + cytosine	4.9	36.2
" + thymine	4	35.7
" + uracil	5.3	36
" + " + cytosine + xanthine	12.5	36.1
Uridine + uracil + cytosine + xanthine + adenine + guanine	16.2	36.2
Uridine + adenine	16.2	39.2
" + guanine	16.1	38
" + hypoxanthine	18.2	39.5
" + xanthine	14.3	39.4
" + adenosine	16	37.4
" + guanosine	15.8	38.2
" + adenylic acid	12.2	39.2
" + guanylic acid	13.9	39.6

* Uridine added at a level of 2 μg per tube (approximately 8×10^{-7} molar).

The growth responses obtained with uridine in combination with purines and pyrimidine bases are listed in Table II. It is apparent during early growth (16 hours at 30°C) that all the purine compounds tested show a synergistic action in the presence of uridine and increase growth significantly above that ob-

* Cytosine supplied by Dougherty Chemicals, orotic acid supplied by Sharp & Dohme; all others supplied by Schwarz Laboratories.

TABLE III. Comparative Growth Responses of *S. faecalis* AB to Varying Concentrations of Uridine and Orotic Acid. 16 hr incubation at 30°C.

Supplement	μg/tube	Growth: optical density × 100
Uridine	2	16.2
"	1	12.7
"	.2	4.4
Orotic acid	10	16.4
" "	5	11.8
" "	1	4.5

tained with uridine alone. No synergism was observed with the pyrimidine bases tested.

Orotic acid can replace uridine although it is less active than uridine as shown in Table III. The amount of orotic acid required for equivalent growth is approximately 8-fold greater than uridine on a molal basis.

Discussion. Since *S. faecalis* AB maintains approximately 30% maximum growth at 24-hour incubation when repeatedly subcultured in a synthetic medium containing no purines or pyrimidines, it appears that the organism can synthesize these compounds at limiting rates.

It has been demonstrated that pyrimidine ribosides and ribotides increase the rate of growth of the test organism, while the free bases with the exception of orotic acid, are not utilized for growth. It has also been shown that purines further increase the rate of growth in the presence of uridine. This synergism is contrary to the findings of Loring and Pierce(9) who observed that the growth of a mutant strain of *Neurospora* requiring uridine or cytidine was inhibited by a molal equivalent quantity of adenosine.

Accepting the general view that purines and pyrimidines are incorporated into nucleic acid by separate metabolic pathways(10-12), the synergism between purines and pyrimidine ribosides or ribotides suggests that growth is initially limited by the slower rate of uridine synthesis, and that the increased growth rate in the presence of adequate uridine again becomes limited by inadequate synthesis of purines.

Hammarsten and coworkers(13,14) and Mitchell and Houlahan(15,16) have postulated alternative pathways of pyrimidine synthesis. Using relative activity or utilization of a metabolite as a basis for postulating the sequence of intermediates in biological synthesis, the data presented here indicate that orotic acid is a precursor of uridine. Similarly, the observation that uridine is most readily utilized(1) suggests that it is the immediate precursor for nucleic acid pyrimidines.

Paege and Schlenk(17) have discussed possible reaction schemes involving orotic acid in pyrimidine metabolism. They were unable to demonstrate either direct decarboxylation of orotic acid to uracil or the formation of orotic acid riboside by enzymes of *Escherichia coli*. In our work with *S. faecalis* AB, it appears that orotic acid (or its riboside) is the probable intermediate decarboxylated since uracil cannot replace either orotic acid or uridine. A recent publication by Michelson *et al.*(18) reporting the isolation of orotic acid riboside from *Neurospora* gives further support to the theory that the biosynthesis of uridine, in some species at least, does not involve a direct coupling of ribose to uracil.

Summary. (1) A study was made of the purine and pyrimidine requirements for maximum rate of growth of a strain of *Streptococcus faecalis*. (2) Orotic acid, though less active, will replace uridine in promoting optimum early growth. (3) All of the purine compounds tested showed no growth stimulation *per se*, but provided a synergistic increase in growth rate in the presence of uridine. (4) The implications of these results for pyrimidine metabolism are discussed.

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Lack of Effect of Cortisone on Inhibitory Action of Antigonadotropic Sera. (19200)

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Numerous clinical and laboratory reports have indicated that cortisone and pituitary adrenocorticotropin (ACTH) may affect normal immune responses by altering the normal interaction between antigen and antibody(1-4).

The formation of an antigonadotropic substance in the serum of patients and laboratory animals after prolonged injection of pituitary gonadotropic extracts has been extensively studied(5). The exact relationship between this antigonadotropic substance and other types of antibodies is as yet obscure(6). In view of the recent observations suggesting an alteration in antigen-antibody reaction by cortisone, we have tested the effect of this compound upon the inhibitory properties of potent antigonadotropic sera.

Several batches of a concentrate of hog pituitary gonadotropin, prepared according to the method of Meyer and McShan(7), were employed as antigen. Young female sheep were injected subcutaneously daily with 200 mg of this powdered antigen in saline suspension for 700 days. Serum was prepared in the usual manner and stored in the frozen state until used.†

The same pituitary preparations used as antigen were also employed in testing the inhibitory potency of the sheep serum. For this purpose, weanling female rats of the Holtzman strain were given subcutaneously 3.3 or 5 mg of the antigen in 0.5 cc water daily for 3 days. A saline suspension of cortisone acetate (0.25 cc) was administered subcutaneously daily for 5 to 11 days beginning 2 to 8 days before the administration of the antigen or antiserum. The antisera were administered subcutaneously daily at a dosage of 0.5 cc or 1 cc as indicated in Table I. All animals were autopsied 24 hours after the last injection.

The data in Table I show that both sheep sera employed were highly effective in suppressing the gonadotropic response in the test rats. The addition of cortisone, even when administered for as much as 8 days prior to the antisera did not reduce the inhibitory effect of the antigonadotropic sera. Thus, there appeared no interference with expected interaction between antigen and antiserum as suggested by other studies.

Summary. Cortisone administration did not interfere with the antigonadotropic effectiveness in the rat of inhibitory sheep sera

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† We are very much indebted to Dr. W. H. McShan for the preparation of the antigen and to the Research Staff of the Upjohn Co. for the preparation of the sheep antisera.

TABLE I. Effect of Cortisone on Inhibitory Action of Antigonadotropic Sera.

Series	Total dose of pituitary antigen* (mg)	Daily dose of antigonadotropic sera (cc)	Daily dose of cortisone acetate† (mg)	Ovarian wt (mg)
A	0	0	0	14 ± 3
	10	0	0	38 ± 6
	10	1	0	12 ± 2
	10	1	.5	13 ± 3
B	0	0	0	17 ± 3
	10	0	0	54 ± 11
	10	1‡	0	18 ± 3
	10	1‡	1	26 ± 7
	10	1§	0	21 ± 2
	10	1§	1	30 ± 5
C	0	0	0	15 ± 3
	15	0	0	28 ± 4
	15	1‡	0	14 ± 4
	15	1‡	.75	13 ± 3
	15	.5‡	0	12 ± 2
	15	.5‡	.75	13 ± 2

* The antigen preparation used in Series C was less potent than that used in Series A or B.
† Cortisone given for 11 days beginning 8 days before antigen in Series A and for 5 days beginning 2 days before antigen in Series B and C. All animals were 23-29 days of age at autopsy. ‡ Sheep serum #5. § Sheep serum #13.
Series A includes 15 animals per group and Series B and C include 6 animals per group.

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Antibiotic Synergism Requires Simultaneous Presence of Both Members of a Synergistic Drug Pair.* (19201)

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Much information is available on the effect of synergistic pairs of antibiotics *in vitro* and *in vivo*, but the mechanism of synergistic action is not known. An understanding of this mechanism might permit more rational and efficient use of drug combinations. A number of theories, not necessarily mutually exclusive, may be proposed to explain synergism, among them the following: (a) The two drugs of a synergistic pair combine chemically to form a compound more effective than either drug alone; (b) one drug kills those organisms that are resistant to the other agent; (c) one drug acts principally on multiplying bacteria, the other on resting organisms; (d) one drug may potentiate the action of another, *e.g.*, by increasing the permeability of the cell wall; (e) potentiation may occur through the simultaneous blocking of alternate enzymatic pathways, *i.e.*, if a given anabolic process essential for growth may proceed by two different

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† Recipient of a Student Fellowship of the National Foundation for Infantile Paralysis.

routes, there may be little or no inhibition of growth by the blocking of one pathway, but complete inhibition by the blocking of both. It seems likely that more than one of these possible mechanisms participate in synergistic effects. Certain ones appear less probable than others. Thus recent work indicates that the members of a synergistic pair of drugs do not interact to form a single compound(1). Synergism has been observed at times when one member of the pair alone had no apparent antimicrobial effect, whereas the combination killed all exposed bacteria(2). In such situations it is unlikely that theories (b) and (c) above play a significant role.

The theories involving some form of potentiation are in agreement with the finding that synergism can be demonstrated in certain pairs of drugs provided at least one of them manifests biological activity(2). If it is acknowledged that synergism may result not from an interaction of two drugs, but rather from the action of a "potentiating" agent on a microorganism which is likewise subjected to another drug, it becomes of interest to determine whether the "potentiating" agent and the active drug must be present simultaneously. It may be that blockage of alternate metabolic pathways would involve simultaneous presence of both members of a synergistic pair of drugs, whereas if a potentiating drug acted through an alteration of cell membrane permeability its effect might persist for some time after its removal. The experiments reported here attempt to differentiate between these possibilities by permitting one drug of a synergistic pair to act for some time, then removing it and substituting the other member of the pair and observing the effect on the viable bacterial population.

Materials and methods. Bacteria. Enterococcus (*Streptococcus faecalis*) strain 16 was tested with penicillin and streptomycin, and *Staphylococcus albus* strain H (Heatley) was tested with terramycin and bacitracin since these combinations were known to be synergistic against these organisms(2,3). Bacteria were grown in proteose peptone No. 3 (Difco) broth and serial dilutions were plated on proteose No. 3 agar. All cultures were incubated at 37°C. *Antibiotics.* Crystalline potassium penicillin G and streptomycin sulfate were obtained from commercial sources. Terramycin hydrochloride (Lot WBW507019) was supplied by Dr. H. H. Anderson, and bacitracin (Lot B-480420) by Dr. L. S. Smith. Stock solutions of all drugs were prepared in 0.85% sodium chloride solution and stored at 4°C. Further dilutions were prepared in distilled water as required. *Technic of test.* Broth containing antibiotic was distributed in 15 ml amounts in large, metal-capped test tubes containing a small amount of sterile sand to aid in resuspending the bacteria after centrifuging. Each tube was inoculated with 0.75 ml of an 18-hour broth culture of the organism under test, to give an initial concentration of 1 to 5 x 10^7 viable organisms per ml. All tubes, including suitable controls, were incubated at 37°C for 4 hours. The number of viable organisms was then determined by plate count and the tubes centrifuged for 20 minutes at 3000 rpm. The supernatant was removed and the sediment washed once in broth warmed to 37°C. The sediment was then resuspended in the original volume of warm broth containing antibiotic and incubation at 37°C was continued. These manipulations took approximately 50 minutes. Subsequently the number of viable organisms was estimated by plate counts at intervals indicated in the figures. In several experiments uncentrifuged control tubes were included. Plate counts of these controls did not differ significantly from the centrifuged tubes and indicated that centrifugation, resuspension, and exchange of menstruum did not result in a measurable loss of bacteria. A typical experiment included the following tubes:

Tube No.	Drug present in 1st 4 hr, μg	Drug present after centrifugation and washing, μg
1	0	0
2	Streptomycin, 100	Streptomycin, 100
3	" 100	0
4	" 100	Penicillin, 3
5	0	Streptomycin, 100
6	Penicillin, 3	Penicillin, 3
7	" 3	0
8	" 3	Streptomycin, 100
9	0	Penicillin, 3
10	Penicillin, 3, + streptomycin, 100	Penicillin, 3, + streptomycin, 100

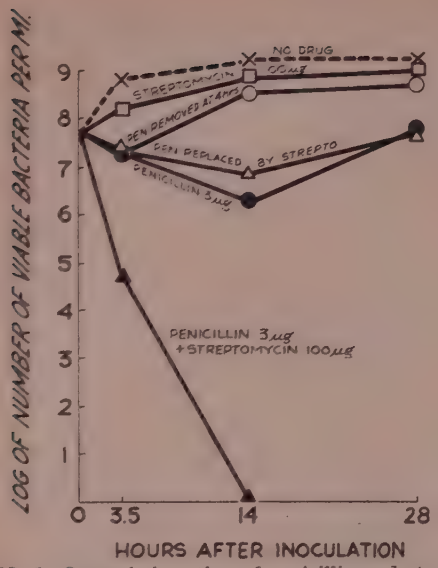

FIG. 1. Synergistic action of penicillin and streptomycin on enterococci. Rapid bactericidal effect when penicillin 3 μg/ml and streptomycin 100 μg/ml act simultaneously, not when penicillin is present alone 4 hr, then removed, and replaced by streptomycin. Likewise there was no synergism when this sequence was reversed (not shown).

Results. A representative experiment illustrating the action of penicillin and streptomycin on enterococci is shown in Fig. 1. In order to avoid crowding of the figure, the curves of 3, 4, and 5 of the above outline have been omitted. The results with these tubes are entirely parallel to those discussed below. When both drugs were present simultaneously throughout incubation the number of viable bacteria fell rapidly to zero. Synergism of the drug pair was evidenced by the great increase in bactericidal rate over that of either penicillin or streptomycin alone. The concentration of streptomycin employed (100 μg/ml) had little apparent effect when acting alone. Penicillin (3 μg/ml) caused only a moderate and temporary decrease in the viable bacterial count. Synergism was not evident when either penicillin or streptomycin acted on the bacteria for 4 hours and then was removed and replaced by the other member of the pair. Unless the two drugs were present simultaneously in significant concentrations synergistic action failed to occur.

Fig. 2 illustrates typical results obtained with terramycin and bacitracin acting on staphylococci. Alone, bacitracin (1.5 units/ml) had a barely noticeable effect, whereas terramycin (1.0 μg/ml) exhibited transient bacteriostasis only. When both drugs acted simultaneously the rate of bactericidal action was sharply increased and the entire population was killed. No such striking synergism could be observed when the two drugs were permitted to act in sequence.

From these results it was concluded that synergism as defined by a rapid rate of bactericidal action and complete killing of the exposed bacterial population could occur only when both members of a synergistic pair of drugs were present simultaneously, not when one preceded the other, and was subsquently removed from contact with the bacteria. In the latter case, there was sometimes no evidence of additive action whatever. In some instances, however, *e.g.*, when terramycin was replaced by bacitracin (Fig. 2), or when penicillin was followed by streptomycin, multiplication of bacteria was inhibited for a longer

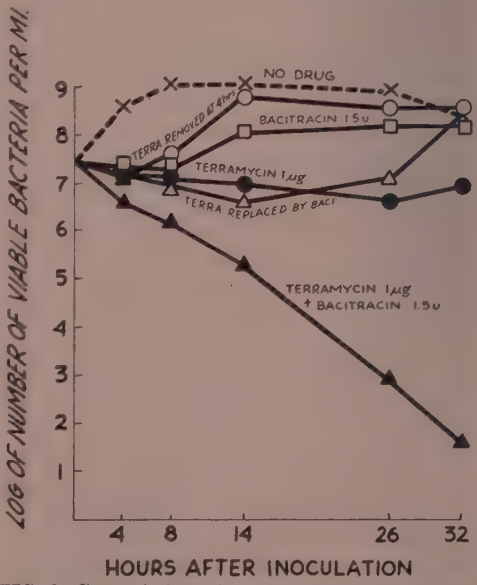

FIG. 2. Synergistic action of terramycin and bacitracin on staphylococci. Marked bactericidal effect when terramycin 1 μg/ml and bacitracin 1.5 units/ml act simultaneously, not when terramycin is present alone for 4 hr, then removed and replaced by bacitracin. Likewise there was no synergism when this sequence was reversed (not shown).

period of time than if the first drug was simply removed and the bacteria permitted to multiply in drug-free broth. That this effect might be attributed to summation (though not synergism) of drug action (perhaps attributable to incomplete removal of the first drug) was suggested by the following experiment. At the time of centrifugation an untreated broth culture was diluted so as to approximate the bacterial concentration of the tube in which the first drug had acted for 4 hours. When a drug was added to this smaller number of untreated bacteria, the rate of multiplication was somewhat more rapid than that of organisms exposed to a sequence of drugs.

This experiment indicates that the additive, though never synergistic effect of two drugs in sequence, can not be attributed to the smaller number of microorganisms acted upon by the second drug. It has not been ruled out that this minor effect could be the result of small, subinhibitory concentrations of the first drug remaining adsorbed on bacteria after washing.

It has been reported that a sudden increase in bactericidal rate followed the addition of streptomycin to an enterococcal population previously exposed to penicillin action for 24 to 72 hours(3). It was similarly observed that in another synergistic drug pair acting on staphylococci, the addition of bacitracin at intervals of 2 to 6 hours to a population exposed to terramycin (or vice versa) resulted in a prompt increase in the rate of bactericidal action. Here, too, even though both drugs were not added initially, simultaneous exposure of microorganisms to both members of a synergistic drug pair resulted in prompt synergism.

Summary and conclusions. (1) It has been demonstrated in two systems that antibiotic synergism is observed only when both members of a synergistic pair of drugs are acting simultaneously on the bacterial population, not when they act in sequence. It may, therefore, be suggested that antibiotic synergism is not usually the result of a modification in a bacterial population induced by one drug, which renders the microorganisms more susceptible to the other drug of a synergistic pair. (2) These findings would tend to favor a hypothesis of synergism involving simultaneous blocking of alternate enzymatic pathways.

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A Quantitative Method for Study of Phenolsulfonphthalein (PSP) Concentration by Surviving Renal Slices.* (19202)

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The prevailing method(1) for studying the transport of phenolsulfonphthalein (PSP) by renal tubular cells *in vitro* is a visual one. The concentrations of PSP, substrates or inhibitors are varied and the ratio of concentrations of any two of these variables required to produce an all or none effect (visible concentration or no concentration) is noted by microscopic examination. The technic employed by Cross and Taggart(2) to investigate the tubular transport of p-aminohippurate (PAH) by surviving renal cortical slices of the rabbit suggested that a similar procedure might be used in *in vitro* studies on the renal tubular transport of PSP, providing a satisfactory method were available for the quantitative estimation of this substance in

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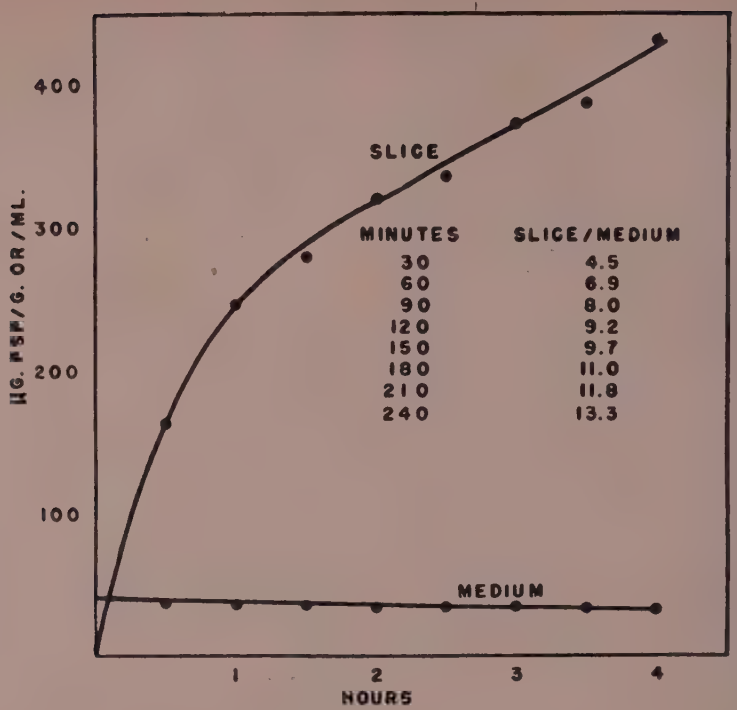

FIG. 1. Effect of incubation time on uptake of PSP by guinea pig kidney slices.

the tissue. Such a method would allow for accumulation of considerably more information with expenditure of less effort than that involved in the technic presently employed.

A technic, described previously in a preliminary report(3), was devised for the estimation of PSP in renal slices and although this yielded satisfactory results it was rather time consuming. A more rapid method designed for this determination is reported herewith.

Methods. Kidneys from guinea pigs were employed in all experiments. The animals were killed by a blow on the head, the kidneys removed, stripped of their capsule and placed in an ice-cold medium of the same composition as that in which they were subsequently incubated, except that the PSP was omitted. Each kidney was quartered and sliced as described by Umbreit, Burris and Stauffer(4), only 3 slices being prepared from each quarter. The slices were then placed in 20 ml beakers containing 5 ml of the buffered medium described by Cross and Taggart(2) and containing 1.06×10^{-4} M (4 mg %) PSP instead of PAH. Care was taken to distribute the slices such that an equal number from each slice level was placed in each beaker. The beakers were placed in a Dubnoff metabolic shaking incubator and shaken at about 100 cycles per minute in an oxygen atmosphere at 25°C. The incubation time was 1 hour and the tissue weight approximately 100 mg unless otherwise stated. After incubation, the beakers were placed in an ice bath and as rapidly as possible the slices removed, blotted and weighed on a torsion balance. For the determination of PSP concentration in the slices, the weighed tissue was transferred to a Potter-Elvehjem homogenizer(5) and homogenized in 11 ml of distilled water. The proteins were precipitated from a 10 ml aliquot of this homogenate by the addition of 1 ml of 10% sodium tungstate followed by 1 ml of 0.67 N sulfuric acid. After mixing and centrifuging, the supernatant was decanted and the precipitate washed twice with 5 ml portions of distilled water. The supernatant and washings were combined and evaporated to dryness in a boiling water bath.

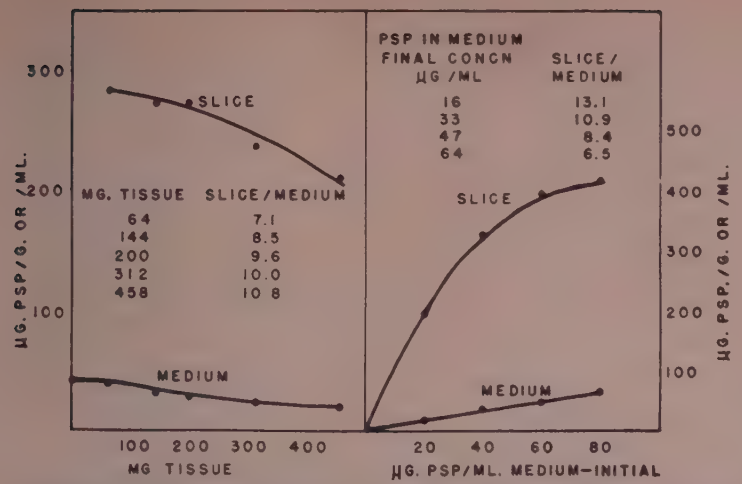

FIG. 2. Influence of tissue wt (left) and PSP concentration (right) on uptake of PSP by guinea pig kidney slices. Along the ordinates are plotted final concentrations of PSP per g of slice or per ml of medium.

Ten ml of 0.1 N sodium hydroxide was added to the dry residue and the concentration of PSP determined in a Beckman spectrophotometer at 564 mμ, using as a reference solution 0.1 N sodium hydroxide. Recovery of known amounts of PSP, added to the renal slices before homogenization, ranged from 97% to 99.5%. After removal of the slices, the medium was centrifuged and 0.4 ml of the supernatant added to 10 ml of 0.1 N NaOH. The concentration of PSP was estimated spectrophotometrically as above.

Results and discussion. In Fig. 1 are portrayed the data illustrating the effect of varying the time of incubation on the uptake of PSP by the renal cortex slice of the guinea pig. The ratio of concentration of dye in the slice to that in the medium (S/M ratio) increased progressively throughout 4 hours. The curve is very similar to that obtained by Taggart(6) for the uptake of PAH from a phosphate buffered medium by the renal slice of the rabbit. Beyer *et al.*(1) have used a bicarbonate buffer for studying the visible concentration of PSP by guinea pig renal slices at 37°C. Employing this bicarbonate buffered medium and a temperature of 37°C we have found that PSP uptake is poor. Maximum concentration occurred in approximately fifteen minutes and on longer incubation the S/M ratio decreased.

The effect of increasing the concentration of PSP in the medium on uptake of dye by the slice is shown in Fig. 2. At low concentrations of PSP in the medium higher S/M ratios are established than at higher concentrations. However, at these low concentrations accuracy of determination of the S/M ratio is sacrificed to some extent.

Cross and Taggart(2) have stated that the amount of tissue employed in their studies on accumulation of PAH by the rabbit kidney slice could be varied between 200 and 400 mg without appreciably affecting the S/M ratio. A similar situation appears to exist when one studies accumulation of PSP by the renal slice of the guinea pig (Fig. 2). Although a progressive increase in S/M ratio is noted as the amount of tissue is increased, above 200 mg this is not great.

Summary. A method has been described for the quantitative extraction and spectrophotometric estimation of phenosulfonphthalein (PSP) in renal cortex slices of the guinea pig. The effect of alterations of time of incubation, concentration of the PSP in the medium, and weight of tissue on the *in vitro* uptake of PSP by this preparation have been investigated. Uptake of PSP increases progressively with increases in incubation time. Increasing the concentration of PSP in the medium results in a decrease in the ratio of

dye concentration in the slice to that in the medium. Ability of the slice to concentrate PSP from the medium is not markedly influenced by alterations of tissue weight between 200 and 400 mg.

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Effect of 6n-prophylthiouracil on Lethal Seizures in Mice.* (19203)

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The antithyroid activity of thiouracil has been often demonstrated and is generally accepted. On the theory that functional inhibition of the thyroid gland would produce decreased spontaneous activity, thiouracil was used to produce a reduction in susceptibility of an inbred strain of mice to sound-induced lethal convulsive seizures.

The author previously found that *age* played an important role in the susceptibility to sound-induced lethal convulsive seizures in tests made on several different inbred strains of mice(1-3). Since the endocrine and physiological levels vary throughout the life span of an individual it was believed that age and metabolism might influence the susceptibility to stress. This was found to be true and, in particular, was demonstrated in the DBA, or inbred dilute brown strain of mice where the highest susceptibility to lethal convulsive seizures at the sound of an electric bell was at the adolescent ages 25-39 days(4). Beyond 80 days of age there were no cases of convulsive individuals. Both sexes were affected, the males slightly more so. The form of convulsive seizures which occur in the DBA strain of mice simulates the grand mal epileptic seizures in humans and it is interesting to note that medical men as Lennox(5) have found the incidence of epilepsy to be greater among children than adults and that the sexes are equally affected. The mouse seizures are of clonic-tonic form and are usually fatal at the peak of susceptibility in the DBA strain.

Methods.† The strain of mice used for these experiments is the dilute brown, DBA/2 Jax, maintained by brother to sister mating. The animals came from the author's colony and are derived from over 200 generations of inbreeding. Two hundred and one animals were used in this experiment; 151 as test or experimental animals and 50 sibs as direct controls. In addition the established performance of the strain as a whole(4) could be used for comparison. Both sexes were used in equal numbers. Seizure susceptibility tests were made at 30 days of age at which age mice are not sexually mature. Thiouracil treatment was started at 21 days of age when the mice weighed about 12 g each. The drug was administered in the food for 9 days previous to the test for seizures, since administering the drug through the drinking water did not prove practicable. The 6n-prophylthiouracil used was obtained from the Overbrook Chemical Co. (Philadelphia). This compound is one of the most highly active antithyroid agents as shown by Astwood(6). Purina Laboratory

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† The author wishes to thank Miss Marie Monastra and Mr. Sidney Chason for their valuable technical assistance.

TABLE I. Effect of 6n-prophylthiouracil on Sound-Induced Lethal Convulsions in DBA/2 Mice at 30 Days.

	No. of animals	% of mice showing convulsions: Standing spasms	Clonic-tonic	Total	% of fatal convulsions
Normal animals*	1316	.0	90	90	73†
Sibs of treated animals	50	.0	94.1	94.1	78
Thiouracil treated	151	31.5	.5	32	.0

* From Vicari, 1951.

† Based upon No. of convulsing subjects.

Chow nuggets were ground very fine and 100 mg of the compound was evenly distributed and mixed in 100 g of the ground chow food. From this mixture nuggets were made and stained with red food coloring to distinguish them from the ordinary nuggets given the controls. Also the shape of the drugged nuggets was made different from the ordinary ones. Both sets of animals were fed in the same way. Their food hopper was filled with their respective nuggets and water was available *ad lib*. It was estimated roughly that the daily food intake was approximately 3 g of food and 5 mg of thiouracil per mouse. The procedure of testing and inducing the convulsions by sound was that described previously by Vicari(4). The intensity of the sound used to induce seizures was approximately 90-93 decibels. One stress test was given each animal.

Results. The mice tested after 9 days feeding with 6n-prophylthiouracil gave no lethal seizures and 68% of the individuals had no convulsive seizures of any degree. The 32% of the individuals which had some form of convulsion did not show the severe clonic-tonic convulsive seizure exhibited by the controls. The per cent of mice showing the different patterns of convulsions in the controls and treated animals are given in Table I.

In the 68% of the treated animals not exhibiting any convulsions, the pattern of behavior was a "sit-and-walk" form in 54% of the individuals, and the "run-and-stop" form in 14% of the individuals. The typical behavior of a treated animal at the sound of the bell was as follows, in succession: the startle reaction, at 8 seconds the mouse circled around, at 16 seconds it stopped, and then sat, at 33 seconds it started running fast, at 45 seconds it began to tremble, quivering all over the body, then at 58 seconds it jumped high, circled, tried wall jumping. At 80 seconds it stopped and sat breathing fast and quivered or sat and groomed. If no convulsion occurred within two minutes the observation was terminated.

In the 32% of the individuals convulsing, the duration of the convulsive spasm was lessened and the preconvulsive period was lengthened as compared with the controls. About one half of the mice, undergoing some form of convulsion, showed a peculiar behavior following the spasm. The animal went into a sort of cataleptic posture and remained so for from 2 to 3 minutes. The other half of the convulsing individuals showed a slightly different behavior. The animal would start to go into a clonic spasm, would *not* fall over on its side but would show a stiffness in the front legs as if paralyzed. This would give its movements a "hopping" gait for several minutes before the normal gait was resumed. Both forms of behavior were observed in both sexes.

A smaller group of 30 individuals which were fed the 6n-prophylthiouracil for 5 days previous to testing at 30 days showed similar protection from lethal seizures as the 9 days treated group.

Discussion. The slight variations in the manifestations of the thiouracil-fed animals may possibly be explained by the fact that the amount of the food ingested by each individual could not be accurately controlled. However, the results *do* show a reduced activity of the animal under sound stress and a very high reduction of susceptibility to clonic-tonic convulsions with total elimination of fatal seizures. That thiouracil has influenced the susceptibility to seizures shows that the thyroid gland has some role in the expression

of seizures and that it may be a contributory factor in its relation to the chief or primary cause.

Thiouracil has been used in morphological experiments but not, as far as can be ascertained in the literature, has it been used for the direct purpose of protecting an animal from convulsive seizures.

Summary. The effects of feeding 0.1% 6n-prophylthiouracil in the food for 9 days to an inbred strain of mice, DBA/2, recognized for its high susceptibility to sound-induced convulsive lethal seizures, were observed. One hundred and fifty-one mice were started the drug feeding at 21 days of age and 30 animals at 25 days. The animals were tested for sound-induced seizures at 30 days when the susceptibility was normally highest. Practically 100% protection from lethal seizures was produced by the use of the drug. Approximately 68% of the individuals showed no form of convulsion. The 32% which had some form of convulsive spasm did not show the severe seizures exhibited by the controls. In this convulsive group the influence of the drug was manifested by a temporary paralysis of the front legs causing hopping movements or the behavior took the form of a trance-cataleptic posture which lasted for 2 to 3 minutes. Also the spasmic convulsion was shortened in duration and the preconvulsive period was lengthened. It may be that the thyroid gland has some important role, even if secondary, in the onset or prevention of convulsive seizures as elicited by sound in the mouse.

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Effect of Bacteriophage on Virulence of *Corynebacterium diphtheriae*. (19204)

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For many years the question as to whether or not virulent (toxigenic) *Corynebacterium diphtheriae* can become wholly avirulent (non-toxigenic) has vexed the public health official and the microbiologist alike. A view widely held for years was that, in persons convalescent from diphtheria, virulent diphtheria bacilli revert to an avirulent state with passage of time. No sound evidence has ever supported this belief. On the contrary, extensive studies(1-7) showed that if the change from the virulent to avirulent state occurs in nature, it must do so very rarely. Until very recently, attempts in the laboratory to alter the virulence of *C. diphtheriae* have failed completely, with the possible exception of the instance cited by Morton(8). No evidence has yet been offered that virulent strains become avirulent in nature.

On the other hand, all views on the stability of *avirulence* of *C. diphtheriae* must now be revised, and all epidemiological considerations concerning diphtheria must be altered in view of the demonstration by Freeman(9) in 1951 that certain bacteriophages readily induce certain strains of avirulent diphtheria bacilli to become definitely toxigenic and virulent for animals. We regard this discovery as one of the most fundamental in the field of diphtheriology since Loeffler's work. Dr. Freeman kindly sent us his avirulent strains and his bacteriophages. Two phages, A_6 and B_6, and five cultures, 324, 325, 326, 334, and 337B, were received. It is to be noted, as a pecu-

liarity of these avirulent strains, that all were isolated from 1 clinically typical case of diphtheria and one unrelated contact. The isolation of avirulent cultures from cases of diphtheria has been previously reported(10). Using these and other materials, attempts have been made to repeat Freeman's experiments in this laboratory. The procedures described by Freeman were modified in these preliminary experiments. Technical details will be described fully elsewhere.

Attempts to isolate any *C. diphtheriae* from the supposedly bacteria-free phage suspensions failed. The 5 strains of *C. diphtheriae* as received from Freeman showed no evidence of virulence. They were plated and about 100 individual colonies were subcultured and tested individually for virulence. None was virulent. Four of the 5 strains were then cultivated in Difco heart-infusion broth in contact with the original phages as received from Freeman. Phage B_6 produced partial lysis of all strains; phage A_6 produced no perceptible lysis. *In vitro* virulence tests made with the broth cultures at this stage showed definite lines indicating virulence of the strains 324, 326, 334, and 337B in contact with phage B_6. The same strains in contact with phage A_6 showed no evidence of virulence at this time. When phage B_6 was applied to young growth of the original strains on agar plates, clear zones appeared in which resistant colonies were observed. Subcultures of these resistant colonies were found to be virulent.

On further culture-to-culture passage of phage A_6, it acquired sufficient potency to produce complete lysis and to induce the change from avirulence to virulence in four of the strains—324, 326, 334, and 337B—in the same manner as phage B_6. In Freeman's laboratory, phage A_6 had not developed this degree of activity. We have also induced these changes in Freeman's avirulent cultures with a phage isolated here by us.

There is no reason not to regard all of these changed strains as true, toxigenic *C. diphtheriae*. Virulence was confirmed in animals.

Attempts to repeat these experiments with strains of avirulent *C. diphtheriae* isolated, not from cases but from healthy carriers, have so far been unsuccessful. However, the change from avirulence to virulence has been readily induced in 2 of our own cultures isolated from what appeared to be clinically typical cases of diphtheria. It is suggested that there may be some significance in the fact that the change may be induced readily in avirulent cultures from cases but not (up to the present) in those from healthy carriers.

Summary. The finding by Freeman that certain bacteriophages can induce certain strains of wholly avirulent *C. diphtheriae* to change into fully virulent, typical *C. diphtheriae,* has been fully confirmed in this laboratory. Preliminary data obtained here suggest that avirulent strains from clinical cases of diphtheria may be more susceptible to this change than strains from healthy carriers.

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Appearance of C-14 in Fumarate and Lactate Following the Injection of DL-Methionine-2-C-14.*† (19205)

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(Introduced by Arnold H. Maloney.)

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Although the glucogenic activity of methionine in phlorizinized dogs was proposed by Vars(1) the relationship between the butyryl part of the molecule and the common intermediates frequently elaborated in the course of the conversion of amino acids to glycogen has not been investigated. It therefore seemed of interest to test the possibility of C-14 appearing in the intermediates of the Krebs cycle when methionine labeled on the alpha-carbon was administered to the intact animal. The present report presents qualitative evidence that C-14 occurs in fumarate and lactate following the intraperitoneal injection of DL-methionine-2-C-14.

Procedure and results. Mice weighing 20 g were injected intraperitoneally with 20 mg (equivalent to 0.04 mc) DL-methionine-2-C-14 suspended in isotonic saline. After 4, 7, and 10 hours, the animals were sacrificed and the livers removed. The procedure for the precipitation of protein and the extraction of organic acids followed that previously described(2). Inert fumaric, succinic, and aconitic acids were added as carriers to the acetone extract and the extracts were chromatographed. The silica gel columns of Isherwood(3) and the procedure of Marvel and Rands for improving resolution on these columns(4) were used. The developing liquid was delivered from a reservoir so arranged that the n-butyl alcohol-chloroform mixture would gradually and automatically increase with respect to the concentration of alcohol (5). Effluent acids were titrated in alternate fractions throughout the chromatogram and fractions not titrated were measured for radioactivity. For chemical identification, fractions near the chromatographic peaks that had not been titrated were measured spectrophotometrically at 230 millimicrons. Since the extinction coefficient of succinic acid is insufficiently high in the ultraviolet to distinguish it from aliphatic acids in general, this acid was examined in the infrared region between 1650 and 1790 cm-1. It showed an absorption pattern identical to that of chemically pure succinate. Lactic acid was identified by the use of p-hydroxydiphenyl(6). The effluent was collected throughout the study by means of the Technicon fraction collector and absorbance was measured on Beckman spectrophotometers Models DU and IR-2. As an index of radio-chemical purity, the degree to which activity followed titrable acidity was determined (Fig. 1). In 2 experiments, each acid was passed through a second chromatographic column. The radioactivity for each acid occurred at the same position and none of the activity was transferred to other por-

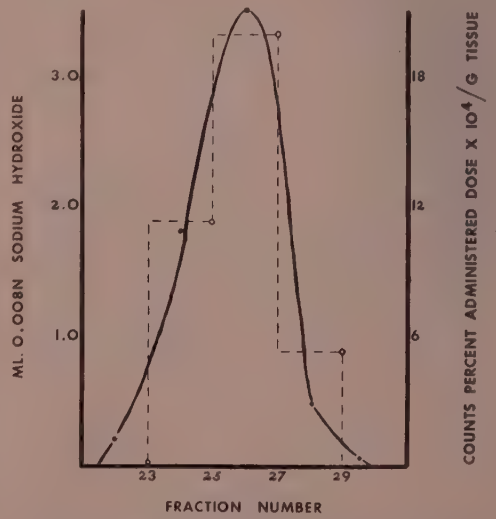

FIG. 1. A comparison of radioactivity and titratable acidity of alternate fractions within the effluent chromatographic zone for the fumaric acid chemically derived from aspartate.

* DL-methionine-2-C-14 was supplied by Tracerlab, Inc.

† Supported in part by a Grant from the Damon Runyon Memorial Fund.

TABLE I. Radioactivity of Certain Organic Acids of Liver Following the Intraperitoneal Injection of DL-Methionine-2-C-14.

Acid	Time after administration 10 hr	7 hr	4 hr
		Counts*	
Fumaric	12.8	24.6	26.4
Succinic-lactic	4	23.8	48.8
Cis-aconitic	5.2	12	7.4

* Expressed as % administered counts $\times 10^4$/g liver.

tions of the effluent. The tissue proteins were examined for labeled aspartic acid. Protein residues from liver and kidney were washed 3 times with 5% trichloroacetic acid containing 1% inert DL-methionine. They were hydrolyzed with 10 times their weight of 6N hydrochloric acid after the addition of 5 mg inert DL-aspartic acid. The freed aspartic acid and its carrier were converted to fumaric acid by means of dimethyl sulfate in alkaline medium(7). The fumaric acid was separated chromatographically. Lactate was degraded by ceric sulfate(8) and the fumarate by acid and alkaline permanganate(9).

The results for liver samples 4, 7, and 10 hours after the administration of the amino acid are indicated in Table I. The succinate and lactate were released together on the silica gel columns, and radioactivity followed titrable acidity with this chromatographic zone. The values shown were corrected for background. The smallest observed count was more than twice the background and the counting error was not greater than 5%. Quantities of wet liver used ranged from 0.8 to 1 g.

Effluent succinate-lactate, as well as fumarate from carcass, showed a similar relationship with respect to acidity and radioactivity. When effluent samples of the succinate-lactate zone from carcass were twice chromatographed with N-amyl alcohol, 30% of the activity was associated with the lactate. No attempt was made to study the aconitate chromatographic zone in this way(1).

Lactate from a single entire carcass was degraded to acetaldehyde(7) and oxidized to acetic acid .75% of the activity of the lactate occurred in the acetate indicating that a large part of the activity was in the non-carboxyl carbons. The results from the degradation(9) of fumaric acid indicated that 52% of the radioactivity was located on the carbon dioxide representing one methine carbon. 48% appeared on the formate derived from one methine along with both carboxyl carbons.

When the fumaric acid derived from aspartate by treatment with dimethyl sulfate was chromatographed(2,3) on silica gel columns, the effluent zone for fumaric acid assayed 74 counts per million counts of administered dose. Progressive changes in titratable acidity accompanied changes in radioactivity within the chromatographic zone for this acid (Fig. 1).

Discussion. The C-14 appears largely in the methine carbons of fumarate and the non-carboxyl portions of lactate. This would seem to indicate that carbon dioxide fixation alone does not explain(10) the path of conversion of DL-methionine to the acids of the Krebs cycle. Two possible pathways of methionine dissimilation have been suggested in the literature. The first describes alpha-amino butyric acid(11) as the intermediate which results on loss of the methyl group of methionine. Deamination of the intermediate occurs *in vivo* (12) but not in enzyme preparations(13). The second pathway postulates that deamination occurs before demethylation. The intermediate gamma-methiol butyric acid, is formed in kidney slices(14) and methyl mercaptan is produced simultaneously.

In the absence of carbon dioxide fixation the metabolic significance of the small amounts of radioactivity found in the organic acids can be evaluated only when the detailed fate of the butyryl part of methionine becomes known. Therefore, we do not attempt in this paper to suggest a pathway by which the C-14 of methionine could appear in lactate or fumarate.

Summary. Qualitative data indicate that C-14 appears in fumarate and lactate following the intraperitoneal injection of DL-methionine-2-C-14.

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Effect of Cortisone and ACTH on Adrenals in Experimental Diphtheria, Shiga, and Meningococcus Intoxication. (19206)

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Involvement of the adrenal glands is a pronounced feature of the intoxication produced in animals by a number of bacterial toxins and in the course of many bacterial infections.

The possible sparing of the adrenals by cortisone was studied in the case of the toxin of *Shigella dysenteriae* (Shiga) and virulent meningococcus infection in mice, and in guinea pigs given a fatal dose of diphtheria toxin. Because of the interrelationship of ascorbic acid and adrenocortical function, experiments were conducted in which cortisone and ascorbic acid were both administered to the same animal even though mice are known to have the ability, not possessed by guinea pigs, to produce their own ascorbic acid. In addition the effect of ACTH was studied in guinea pigs receiving 1 MLD of diphtheria toxin.

Methods. The cortisone used was the dry acetate or free alcohol which was prepared for use by dissolving a weighed amount in the smallest quantity of acetone required to effect complete solution and then quickly bringing up to full volume with physiological saline solution so that the required dose was contained in 0.5 ml. A drop of "Tween 80" per 100 ml helped stabilize the suspension. The ACTH solution was freshly prepared prior to injection by dissolving a weighed amount of the dried powder in sufficient physiological saline solution so that the required dose was contained in 0.5 ml. Weights of ACTH are expressed in terms of Armour Standard No. LA-1A. The animals used in these experiments were guinea pigs weighing 240-280 g and pure line CFW strain white mice weighing 16-20 g. All guinea pigs were weighed daily. All animals were autopsied at death or after sacrifice.

Effect of cortisone and ACTH on guinea pigs given 1 MLD of diphtheria toxin. The hemorrhagic appearance of the adrenals has long been cited as the most conspicuous finding in guinea pigs dying from diphtheria toxin. The course of intoxication following the administration of 1 MLD of diphtheria toxin has been closely observed in this laboratory for a great many years, an experience which provided a background for the detection of any deviation from the expected course which could be attributed to cortisone, cortisone plus ascorbic acid, or ACTH. In 3 experiments all guinea pigs were given 1 MLD of diphtheria toxin subcutaneously. In Exp. 1 and 2 ACTH was given twice daily (0.17 mg) or cortisone daily (0.34 mg) for 15 days, or until death. In Exp. 1 the initial dose was given subcutaneously at the same time as the toxin.

In Exp. 2 the ACTH and cortisone injections were begun on the day prior to toxin administration. The guinea pigs in these first 2 experiments were in groups of 8, a similar number receiving the toxin only, as controls. In the 3rd experiment, which included 56 guinea pigs, ACTH was omitted, the daily dose of cortisone increased from 0.34 to 1.54 mg, and 25 mg ascorbic acid given daily to half of the animals in each group. All animals were examined for subcutaneous edema, local necrosis, ascites, and weakness. At autopsy the presence and degree of subcutaneous edema, subcutaneous hemorrhage, pleural and abdominal fluid, and congestion of the adrenals were noted. The term congestion is used in preference to hemorrhage because the histological appearance of the adrenals of both guinea pigs and mice is that of congestion of the vessels rather than hemorrhage(1). No marked differences were noted between any of the 3 experiments, either before death or at autopsy, although animals receiving cortisone had an average death time of 6 days as against 8.5 days for the controls. The course of action of the diphtheria toxin was unaffected. All animals showed the following signs of diphtheria intoxication: Loss of weight, loss of appetite, local edema and necrosis, increase in pleural and peritoneal fluid, and congested adrenals, with no demonstrable difference in degree between control groups and corresponding treated groups.

Effect of cortisone on Shiga intoxication in mice. In recent studies on the effects of the toxins of *Shigella dysenteriae* (Shiga) in monkeys(2), the most conspicuous finding was that of hemorrhage: petechial hemorrhages in the skin, hemorrhage into lymph nodes in many regions, and especially hemorrhages into adrenals and into heart muscle. It seemed worth while to determine whether or not Shiga toxin produced similar hemorrhages in mice, and if cortisone might have any effect upon their occurrence. A preliminary experiment was done with 120 mice, 1/3 of which were treated with cortisone and 1/3 with cortisone plus ascorbic acid. All received Shiga toxin intravenously.

In mice receiving Shiga toxin there is a minimum asymptomatic period of at least 18 hours, often extending to 4 or even 6 days. The signs of intoxication are those of an ascending spastic paralysis(3). The mice in the present experiments were observed for 7 days after injection of the toxin. Neither cortisone, ascorbic acid, nor combination of the 2 appreciably lengthened the life of the mice. Ten mice died within 48 hours and were autopsied. Of these, 2 had received cortisone, 4 were controls that received toxin only, and 4 had received toxin and ascorbic acid. Three of the 10 had intensely red adrenals; 2 of these were controls and one had received toxin and ascorbic acid.

In a second similar experiment, 2 groups of 30 mice each were used. One group was given 0.5 mg cortisone on each day of the study. The other group received no cortisone. On the second day both groups were given varying doses of Shiga toxin intravenously. All mice dying in this experiment were autopsied. All those surviving the 7 day period of observation were killed and examined. The time of death seemed uninfluenced by administration of cortisone, but fewer of the cortisone-treated mice showed congested adrenals than those receiving toxin alone. This difference, while not great, seemed definite and this point was then examined further in 3 more extensive experiments, summarized together in Table I.

Those surviving 7 days were sacrificed. All mice were autopsied and the adrenals examined. Many adrenals were bright red, corresponding in appearance to those of the guinea pigs dying from the effects of diphtheria toxin. The appearance of the adrenals was recorded according to intensity of congestion as *red, pink,* or *pale.* The results indicated that cortisone did appear to exert a sparing effect on the adrenals as judged by the degree of congestion observed. More than twice as many untreated animals showed marked congestion as compared with those receiving cortisone.

Effect of cortisone on meningococcus toxin and infection in mice. Massive hemorrhage into the adrenals is the most conspicuous finding at autopsy in patients dying with the Waterhouse-Friderichsen syndrome. Until fairly recently such overwhelming meningo-

TABLE I. Effects of Cortisone on Mice Receiving Varying Doses of Shiga Toxin.

Inoculum of Shiga toxin	Total No. mice T*	Total No. mice U*	Deaths T	Deaths U	Adrenals: Red T	Red U	Pink T	Pink U	Pale T	Pale U
.5 ml of 1/25	10	10	9	9	0	4	9	6	1	0
1/50	20	17†	16	15	4	11	11	6	5	0
1/100	30	30	20	28	5	12	13	14	12	4
1/200	20	25	9	14	3	7	5	6	12	12
1/300	10	20	3	8	3	3	2	4	5	13
1/400	0	14	—	4	—	4	—	3	—	7
Total	90	116	57	78	15	41	40	39	35	36
% T or U			63	67	17	35	44	34	39	31

* T = treated mice; U = untreated mice.

† Animals consumed by others in the group not counted.

Combined results of 3 exp., 60 mice each. 30 in each exp. given .5 mg cortisone daily subcut. On 2nd day all mice given graded doses of Shiga toxin BS-24 intravenously. Additional groups of normal mice—same age and wt—given higher toxin dilutions to confirm LD_{50}.

coccus infections, with the accompanying intoxication, were considered invariably fatal. Within the last few years, a number of recoveries have been reported(4-12). These have been attributed to combination therapy with sulfonamides, antibiotics, large amounts of fluid given intravenously, and whole cortical extract.* The possibility of producing a fatal intoxication in mice and of studying the effect of cortisone upon such a condition was investigated. Meningococcus "endotoxin" was prepared from a heavy suspension of 18 hour agar growth of a mouse-virulent Group I meningococcus (NIH 1027). Titration by intraperitoneal injection into mice showed the LD_{50} to be 0.5 ml. Mice dying from this amount of toxin showed general hyperemia and varying degrees of injection of the axillary and inguinal blood vessels. The adrenals were bright red. Injection of larger amounts of the toxin was followed by conspicuous hemorrhage into the lymphatic tissue of the intestinal walls.

This meningococcus "endotoxin" was unreliable. Either the intoxication seemed to be overwhelming or else it was not constantly fatal. Production of meningococcus infection in mice promised a more consistent picture. This same strain of meningococcus (NIH 1027) was suspended in 3% mucin and titrated by intraperitoneal injection into mice. The LD_{50} was found to be approximately 20 organisms. The mice that died in this titration all showed bright red adrenals, much hyperemia, injection of blood vessels, and usually hemorrhage into the axillary and inguinal lymph nodes, a picture closely paralleling that produced by the "endotoxin." The appearance of the adrenals was similar to that in mice receiving fatal doses of Shiga toxin. In a histological study of the adrenals of mice infected with meningococci, Ruml and Bohnhoff(13) found depletion of fat, engorgement of blood vessels and inflammatory cell infiltration.

In the experiment summarized in the upper portion of Table II, there was an apparent tendency for the adrenals to be less involved, as judged by the degree of congestion, in those animals to which cortisone was given. This experiment was repeated, using another strain of a different serological group of meningococcus (NIH 2103) Group II. Titration in 5% mucin indicated an LD_{50} of 2-10 bacteria. Results are shown in the lower portion of Table II. Strain NIH 2103 affected the adrenals less than did strain NIH 1027, but here again some sparing action seems to have taken place in the mice receiving the cortisone. The excess of ascorbic acid seems to have been without effect in either case.

With both of these strains excessive doses of culture were used. Possibly a more obvious sparing action may have been evident had smaller inocula been given. A further

* Since this paper went to press, two clinical reports of the successful treatment of Waterhouse-Friderichsen syndrome with cortisone have appeared; Nelson, J. and Goldstein, N., *J.A.M.A.*, 1951, v146, 1193; Newman, L. R., *Ibid.*, v146, 1229.

TABLE II. Effect of Cortisone and Ascorbic Acid on Mice Receiving Varying Doses of Meningococci in Mucin.*

Inoculum (LD_{50})	No. mice	Deaths CA†	Deaths C†	Deaths O†	Appearance of adrenals: Red CA	Red C	Red O	Pink CA	Pink C	Pink O	Pale CA	Pale C	Pale O
Exp. 1													
(NIH 1027) 100000	30	10	10	9	1	0	3	8	6	5	1	4	2
10000	30	10	9	10	0	0	8	2	6	1	8	4	1
Total	60	20	19	19	1	0	11	10	12	6	9	8	3
%		100	95	95	5	0	55	50	60	30	45	40	15
Exp. 1													
(NIH 2103) 10000	30	10	10	9	1	3	8	2	0	0	7	7	2
1000	30	10	10	8	3	2	6	0	2	1	7	6	3
100	29	10	10	8	2	4	8	2	3	1	6	3	0
Total	89	30	30	25	6	9	22	4	5	2	20	16	5
%		100	100	86	20	30	76	13	16	7	67	53	17

* Exp. 1—3 groups 20 mice each. 1 group received 1 mg ascorbic acid and .5 mg cortisone daily. 1 group received .5 mg cortisone daily. 1 group received neither cortisone nor ascorbic acid. On 3rd day all mice given from 1000 to 100000 LD_{50} of 5 hr culture of meningococcus NIH 1027 (I) in 1 ml of 3% mucin intraper. Exp. 2—3 groups of 30 mice each. Cortisone and ascorbic acid given as in Exp. 1. On 3rd day all mice given from 100 to 10000 LD_{50} of 5 hr culture of meningococcus NIH 2103 (II) in 1 cc of 5% mucin intraper. All animals autopsied.

† C, cortisone; A, ascorbic acid; O, mice receiving neither ascorbic acid nor cortisone.

series of experiments designed to demonstrate more fully the tendencies indicated above was next carried out using 2 Group I strains, NIH 1027 and NIH 1966. These were arranged in the same manner except that ascorbic acid was not administered. The infecting meningococci were suspended in 3% mucin and the doses varied from 5 LD_{50} to 10000 LD_{50}. Of 60 treated animals receiving strain NIH 1966 the numbers showing red, pink, and pale adrenals were 20, 31, and 9, while for 60 receiving no treatment the figures were 38, 18, and 4. In the case of 30 treated and 30 untreated animals receiving strain NIH 1027 the corresponding figures were 12, 9, 9, and 20, 6, 4. Thus fewer animals receiving cortisone showed congestion of the adrenals as compared with corresponding animals receiving the same dose of organisms but no cortisone. Cortisone did not affect the mortality.

Conclusions. (1) Neither ACTH, cortisone, nor cortisone in conjunction with ascorbic acid appeared to have any effect on the outward course of intoxication of guinea pigs given lethal doses of diphtheria toxin. (2) Neither cortisone nor cortisone in conjunction with ascorbic acid affected the mortality of mice receiving fatal doses of either Shiga toxin or meningococci suspended in mucin. (3) Cortisone appeared to exert a protective action on the adrenals of mice receiving fatal doses of Shiga toxin and meningococci suspended in mucin, as judged by the degree of congestion.

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Antithrombic Activity of Chicken Plasma Induced by Estrogenic Substances. (19207)

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This report deals with observations which indicate that the blood plasma of chickens under estrogenic stimulation exhibits heparin-like antithrombic activity, and that this phenomenon can be demonstrated readily under naturally occurring and experimental conditions. During the course of blood coagulation studies employing chicken plasma, it was noted that plasma from pullets approaching maturity clotted very slowly with amounts of thrombin ordinarily used to defibrinate oxalated plasma. It was further observed that some of the slow-clotting plasmas were lactescent or lipemic in appearance, and that the observed antithrombic effect was exhibited only during the stage of preparation for egg-laying and the actual period of egg production. The plasma of immature fowls of either sex, males or capons of all ages, and moulting or broody hens showed normal reactivity with thrombin solutions.

Efforts were then directed toward experimental production of this state of increased antithrombic activity. It was found that the condition could be induced consistently in chickens of any age and of either sex by the parenteral administration of synthetic estrogenic compounds. Several commercially available preparations were used with similar results, although the drugs employed showed somewhat different potencies. The dosage necessary to cause the change in blood clotting behavior also caused lipemia, oviduct growth, and other changes associated with estrogenic stimulation.

Materials and methods. The chickens were obtained from a local commercial hatchery, and were maintained on a standard control laboratory diet(1). Most of the chickens were White Leghorns, but limited numbers of White Rocks and New Hampshire Reds were also used. The estrogenic drugs employed included diethylstilbestrol, hexestrol, and dienestrol.* The given dosage of the drug was dissolved in 1.0 ml of sesame oil or corn oil (Mazola), and injected subcutaneously. Clotting activity was measured in several ways, including whole venous blood clotting time, with and without the addition of thromboplastin, and measurements of the clotting activity of blood and plasma upon the addition of measured amounts of thrombin. Venous blood clotting time was determined as the time necessary for formation of a firm clot in a clean glass test tube at room temperature. The thromboplastin was prepared by saline extraction of chick brain(2). To measure the clotting time with added thromboplastin, 1.0 ml of blood was added to 0.1 ml of thromboplastin. Thrombin from several sources was tested, including commercially prepared bovine thrombin (Parke-Davis), and preparations from chicken, dog, and bovine plasma using modifications of Sternberger's method (3). The essential modifications of Sternberger's technic employed were the substitution of oxalated plasma for whole citrated blood, and the use of beef lung or chick brain in place of human milk as a source of thromboplastin. The final dilution of thrombin used in most determinations contained 5% calcium-free acacia, which served to stabilize thrombin activity. Clotting activity of thrombin was determined by adding 2 volumes of dilute thrombin solution to one volume of oxalated plasma at a room temperature of approximately 25°C. A control clotting time of between 12 and 15 seconds was obtained by appropriate dilution of the thrombin. Blood obtained by jugular venepuncture was drawn into one-seventh its volume of 1.85% potassium oxalate, and chilled with cracked ice until used. The oxalated plasma was separated by centrifugation of blood in an angle centrifuge in a refrigerated (5°C) room. In some instances comparative studies were done employing sodium citrate or purified amberlite resin as anticoagulants(4).†

* Dienestrol was supplied by White Laboratories, Newark, N. J.

TABLE I. Response to Single Injection of Dienestrol.

Days after inj.	Clotting time (% of control) A	B	Visible lipemia A	B
0	100	100	0	0
1	128	227	1+	1+
2	152	272	2+	3+
3	136	680	2+	4+
4	125	267	0	3+
5	102	346	0	3+
6	98	910	0	3+
7	—	247	0	3+
8	100	247	0	2+
9	103	181	0	2+
10	91	167	0	1+
11	114	128	0	Trace
12	80	102	0	0

A single 10 mg inj. of dienestrol was given to each of 2 White Rock cockerels 75 days of age, designated A and B. Clotting times express the ratio of values obtained with oxalated plasma of inj. and control cockerels of the same hatch, using a thrombin solution which clotted control plasma in approximately 12 sec. Degree of lipemia was estimated from the appearance of the plasma samples.

Results and discussion. Increased resistance to the clotting action of added thrombin usually was observed in blood or plasma of the treated chickens beginning 24 to 36 hours after injection of the estrogenic drug. A maximum effect was attained consistently within 72 hours after the administration of adequate dosages. Definite effects were noted in newly hatched chicks given single doses of as little as 2 mg of dienestrol or hexestrol, or 5 mg of diethylstilbestrol. Dosages many times the minimal effective dose did not shorten the time interval necessary for demonstration of the antithrombic effect. The changes in blood clotting behavior followed the same general course as the observed lipemia, but did not exactly parallel this condition. Thus, some of the animals showed altered clotting activity a day before lipemia was observed, while others showed the reverse order of effects. As a rule, the disappearance of visible lipemia coincided with the loss of increased antithrombic activity after estrogens were discontinued.

Table I shows representative results obtained by giving a single injection of an estrogenic compound to immature cockerels. There was considerable variation in response. Chick A showed a sub-maximum response, while the peak response of Chick B was equal to the maximum effect obtained by injecting several times the given drug dosage into other cockerels of the group. Oxalated whole blood reacted in a manner similar to oxalated plasma and comparable data were obtained using sodium citrate or amberlite resin as anticoagulants. Similar results were obtained with all types of thrombin preparations tested.

The venous blood clotting time of estrogenized chickens was greatly prolonged, as shown by Table II. The very slow clotting of chicken blood is greatly accelerated by the thromboplastic activity of minute quantities of contaminating tissue juice. Despite this source of error, the observed difference between the mean clotting times of the control group and the estrogen-stimulated group was more than 4 times the standard error of the difference between the mean values of the two groups. Variability was greatly reduced in the shortened clotting times obtained by adding large amounts of thromboplastin to the blood. The observed difference in this group was more than 6.5 times the standard error of the difference.

The clotting activity could be modified by the *in vivo* or *in vitro* addition of protamine sulfate in a fashion similar to the interaction of this drug with commercial heparin preparations.‡ Table III illustrates the effects produced by addition of protamine to normal cockerel plasma, with and without the addition of heparin, and to the plasma of an estrogen-treated cockerel or a laying hen. The addition of protamine shortened the clotting time of the control plasma. This may have been due to the presence of an appreciable amount of heparin or a heparin-like substance in normal plasma. The effect of added heparin was easily overcome by addition of small amounts of protamine. Much larger amounts were necessary to bring about comparable reductions in clotting time of plasma of the laying hen and the estrogen-treated cockerel. Addition of larger amounts of protamine length-

† Amberlite Resin IR-100, Rohm and Haas Co., Philadelphia, Pa.,

‡ Protamine sulfate was supplied by Eli Lilly & Co., Indianapolis, Ind.

TABLE II. Clotting Time of Venous Blood.

	Control	Estrogenized	Observed diff.	Stand. error of diff.
Venous blood clotting time (min)	29.7	70.6	40.9	9.38
Clotting time with added thromboplastin (sec)	35.6	44.1	8.5	1.25

Venous blood coagulation times are mean values of 31 determinations on control cockerels or capons and 31 determinations on estrogen injected chickens or laying hens. Clotting times with added thromboplastin are mean values for 84 determinations in each group, in controlled series employing equal numbers of immature White Leghorn chickens of both sexes in each group. The treated group received 5 mg of hexestrol daily.

TABLE III. Effect of Protamine Sulfate Added to Plasma Thrombin Reaction Mixture.

Protamine added, mg	Clotting time (sec)			
	Normal cockerel	Normal cockerel + heparin	Laying hen	Estrogenized cockerel
0	14.3	25.2	18.9	27.4
.05	13.6	12.7	15.3	25
.10	9.9	9.2	14.6	20.3
.20	10	8.1	10.3	17.3
.30	16.8	8.6	8.2	14.3
.40	19.6	9.9	7.9	14.3
.50	20	20.5	8.2	13
.75	—	—	12.6	11.7
1	19.5	20	21	8.4
1.5	—	—	14.7	10.7
2	21	20	22	16.9

Clotting mixture consisted of .1 ml. plasma, .1 ml protamine sulfate solution in normal saline, and .2 ml. chicken thrombin solution. Heparin (Abbott) added to normal cockerel plasma was 1 Toronto unit per ml. The estrogenized cockerel had received 10 mg of dienestrol three days before determinations were made.

ened the clotting time of control plasma, and caused an abnormal, flocculent clot. The addition of heparin partially protected the control plasma from the deleterious effects of excess protamine. The plasma of estrogen-stimulated fowl also showed greater tolerance to excess protamine than shown by the control plasma.

No abnormality of other blood clotting factors of the estrogenized chickens was detected. Prothrombin levels were normal by the two-stage method(1). Fibrinogen was present in amounts sufficient to form a firm clot, and the addition of prepared fibrinogen (Armour) did not correct the clotting defect. The formed clot was stable at room temperature over night with no indication of abnormal fibrinolysis. The plasma antithrombin levels were determined by a method similar to that of Klein and Seegers(5). Chicken plasma was incubated for two hours at room temperature with an equal volume of bovine thrombin preparation (Parke-Davis) containing 1500 units per ml. One ml of either the control or estrogenized plasma inactivated approximately 700 units of thrombin under these conditions. The plasma of the estrogen-treated chickens thus was shown to have essentially normal total capacity to inactivate thrombin. This is in contrast to its heparin-like ability to interfere with the thrombin and fibrinogen reaction.

The heparin-like effect produced in the experimental fowl was prevented by agents which interfere with normal metabolism of the injected estrogen. This was demonstrated by the simultaneous administration of estrogenic drugs and known estrogenic antagonists. The folic acid antagonist, 4-amino pteroylglutamic acid (Aminopterin), and the purine antagonist 2, 6-diaminopurine were used in a manner similar to that reported by Hertz(6), and Hertz and Tullner§(7). The effects of

§ Aminopterin was supplied by Dr. J. M. Ruegsegger, Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y., and the 2,6- diaminopurine was supplied by Dr. G. H. Hitchings, Wellcome Research Laboratories, Tuckahoe, N. Y.

a single 5 mg injection of hexestrol or dienestrol were partially or completely prevented by daily subcutaneous injections of 5 mg of Aminopterin or 50 mg of 2, 6- diaminopurine. Results were somewhat variable and the antagonistic drugs were extremely toxic in the amounts given, resulting in death of most of the test chicks between the third and seventh day of drug administration.

Lipemia produced in chickens by oral administration of 2 ml of lard or corn oil per 100 g body weight was not accompanied by significant changes in clotting. Emulsions of plasma and oil were prepared by repeated forcible ejection of the material through a 25 gauge needle attached to a glass syringe. Preparations containing 10% oil and 1 mg of hexestrol or dienestrol per ml clotted normally.

Plasma from estrogenized chickens retained the heparin-like effect after extraction with 15 to 20 volumes of ether, and the fatty residue remaining after evaporation of the ether at room temperature had no antithrombic properties. Transfusions of blood from an estrogen-treated fowl to a normal fowl caused the transfused animal to exhibit lipemia and the typical heparin-like effect when 50% to 75% of its calculated blood volume had been replaced. The inhibition of the thrombin-fibrinogen reaction disappeared within a few hours, whereas the lipemia still was visible 24 hours later.

Attempts to isolate or further identify the substance responsible for the described effects have not been successful to date. Heparin assays by the methods of Monkhouse and Jaques(8) or Gibson and co-workers(9) have yielded negative results. It was found, however, that added heparin was recovered incompletely from control chicken plasma by these methods, and not at all when added to the lipemic plasma of estrogenized chickens in the usual amounts.‖ Recently reported investigation indicates that native heparin in mast cells exists as a lipoprotein complex(10). The above observations suggest that the active principle in estrogenized chicken plasma may be heparin in a combined form from which the heparin moiety is not readily isolated.

Summary. The plasma of laying hens exhibits an inhibitory effect on the interaction of thrombin and fibrinogen. Similar inhibitory activity can be induced in the plasma of chickens of any age and of either sex by the administration of synthetic estrogenic compounds. This effect can be prevented by agents which interfere with normal metabolism of the estrogenic material in the experimental animal. The observed effect is similar to that produced by heparin, but plasma assays have been negative for heparin, and the substance responsible for the heparin-like activity has not been isolated or identified.

‖ We wish to thank Dr. R. B. Gibson and Mr. Saul Green for assistance with heparin assays of plasma.

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Effect of Adrenalectomy on Dehydroascorbic Acid Diabetes.* (19208)

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Although dehydroascorbic acid is a normal cellular constituent it produces diabetes resembling that from alloxan(1-3). Adrenalectomy ameliorates pancreoprive(4) and alloxan diabetes(5) and may benefit dehydroascorbic acid diabetes.

Experimental. Male Sprague-Dawley rats of 110-180 g were injected with 0.2 g per kilo of dehydroascorbic acid followed in 10-30 minutes by 0.8 g per kilo on each of 3 consecutive days. The course of the diabetes was estimated from weekly blood sugar determinations. All the rats used in this experiment developed stabilized diabetes of at least 10 weeks duration. The food and water intake, the urine volume and the urine sugar output (6) were determined for 5 consecutive days and the average blood sugar was obtained by averaging 5 consecutive weekly determinations. Following adrenalectomy the animals were maintained on a 0.8% sodium chloride solution instead of water(4) and again studied as before for 5 consecutive days. A group of non-operated dehydroascorbic acid diabetic rats were maintained on water and then on 0.8% sodium chloride. The administration of salt (Fig. 1) increased the glucosuria, polyuria, polydipsia, and polyphagia, but decreased the hyperglycemia. Following adrenalectomy, however, the hyperglycemia was reduced and the other signs of diabetes were also ameliorated. P values were calculated in two different ways. First, the standard deviations for the various criteria before and after adrenalectomy were compared. Secondly, each criterion was analyzed by comparing the standard deviation of the change produced in switching from water to sodium chloride solution in the adrenalectomized group with the standard deviation of the corresponding change produced in the non-operated group. The P values are shown in Fig. 1 and indicate

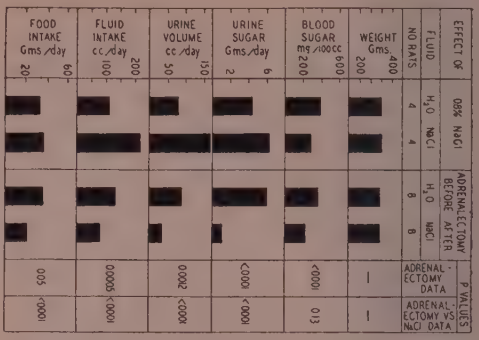

FIG. 1. Effect of adrenalectomy on dehydroascorbic acid diabetes.

that the amelioration resulting from adrenalectomy is statistically significant.

Discussion. The changes produced by the administration of salt are in agreement with earlier work(7). Following adrenalectomy the changes in the diabetic signs are the reverse of those produced by high salt intake in non-operated rats, except that the blood sugar falls even lower following adrenalectomy. Glucosuria, polyuria, polydipsia, and polyphagia are all ameliorated by adrenalectomy in spite of a high salt intake. These results are similar to those obtained after adrenalectomy in pancreatectomized(4) or alloxan injected(5) rats and are consistent with the concept that dehydroascorbic acid diabetes is a pancreatic form of the disease.

Summary. Dehydroascorbic acid diabetes in rats is ameliorated by bilateral adrenalectomy.

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A Study of Moniliasis in Aureomycin Therapy. (19209)

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Numerous reports have appeared in the literature describing the development of moniliasis following antibiotic therapy(1-3). Finally, in April 1951 the Council on Pharmacy and Chemistry of the American Medical Association decided that a warning statement should be added to the label of chloramphenicol, aureomycin, and terramycin(4). This Report of the Council pointed out that the overgrowth of monilia and other yeast-like organisms following the administration of antibiotics occurs most frequently in the large bowel and is of slight importance; however, it emphasized that deaths from pulmonary moniliasis can occur following therapy with these new preparations.

It was suggested to us that the addition of esters of paraben (parahydroxybenzoic acid) to aureomycin might prevent the development of moniliasis(5). These agents have been employed in foods and drugs as preservatives for many years(6,7). The methyl ester of paraben is effective against mold while the propyl ester is active against yeast(7). This paper is a report of the comparative incidence of moniliasis in patients receiving aureomycin and in those receiving aureomycin containing paraben.

Experimental. *In vitro* sensitivity studies using 4 strains of *C. albicans* and 1 strain of *C. krusei* established a definite inhibitory effect by mixed paraben esters. Eighty mg of methyl paraben and 20 mg of propyl paraben were added to 10 ml of physiological saline solution and serial dilutions were prepared. The paraben esters did not completely dissolve which made accurate interpretation of results difficult. The yeast was inoculated on Sabourand's medium containing various amounts of paraben ranging from 10 to 2500 μg per ml of medium. All control cultures having no paraben esters revealed yeast within 24 hours, but those containing 1000 to 2500 μg of paraben per ml of medium required (with a single exception) 96 hours. Following these studies capsules, powder and suppositories were prepared for us by the Lederle Laboratories Division of the American Cyanamid Company. Each of these preparations was supplied in 2 forms—one consisted of aureomycin alone and the other of aureomycin with paraben esters. This investigation involving 186 patients was divided into 3 sections: oral, vaginal and rectal studies.

Oral studies. Fifty-eight patients were selected who had no apparent gastrointestinal disease and had received no antibiotic or chemotherapeutic agent during the previous 72 hours. Thirty were given 500 mg of aureomycin orally every 6 hours, while 15 received the same medication except that each 250 mg capsule of aureomycin contained 142 mg of methyl paraben and 35.5 mg of propyl paraben. Inasmuch as these 45 patients had various infectious diseases, the duration of therapy ranged from 1 to 19 days with the average being 7 days. Thirteen additional patients were given 80 mg of methyl paraben and 20 mg of propyl paraben every 3 hours for 4 days. In each case efforts were made to obtain daily stool specimens; 1 to 3 control specimens were obtained in every instance before beginning therapy. Of the 15 patients who received aureomycin with paraben, 2 revealed *C. albicans* in the stool after treatment; though all had been negative originally. One of these patients noted mild nausea, while the other was asymptomatic. Two additional cases in this group noted nausea and vomiting; mild, painless diarrhea was observed by 2 others. In the group of 30 patients to whom aureomycin without paraben was administered, 21 were found to harbor *C. albicans* in the stools after treatment; though only 6 had revealed yeast prior to therapy. Five cases in this group noted nausea, vomiting or mild diarrhea; but only 1 of 3 patients who developed nausea and vomiting revealed *C. albicans* in the stool. However, yeast was demonstrated in the feces

TABLE I. The Effect of Paraben on the Occurrence of *C. albicans* in Aureomycin Therapy.

	Type of treatment	No. of cases	Cases positive for *C. albicans* Before treatment	After treatment
Oral	A. Aureomycin alone	30	6	21
	B. Aureomycin with paraben	15	0	2
	C. Paraben alone	13	8	3
Vaginal	A. Aureomycin alone	27	5	14
	B. Aureomycin with paraben	57	8	13
	C. Paraben alone	10	1	1
	D. Aureomycin and Propion Gel	15	4	7
Rectal	A. Aureomycin alone	9	1	2
	B. Aureomycin with paraben	10	2	1

of both cases that had diarrhea.

Thirteen patients received 800 mg of the methyl and propyl esters of paraben each day for 4 days. Initially 8 of these cases disclosed *C. albicans* in the stool. At the conclusion of therapy, however, this yeast was present in the feces in only 3 cases. It should be noted that 1 of these patients had not disclosed *C. albicans* prior to paraben therapy. No toxicity to treatment was observed. The results obtained in the oral studies are summarized in Table I.

Vaginal studies. Only patients who had not received an antibiotic or chemotherapeutic agent for 72 hours were included in this investigation. Vaginal cultures for yeast were obtained prior to treatment and then on the second, fifth and eighth days. In 7 cases a 250 mg gelatin capsule of aureomycin was inserted into the vagina each day for a week. In 6 additional patients, the same procedure was carried out except that Propion Gel (calcium propionate 9.5%, sodium propionate 9.5% and propionic acid 1%; Wyeth) was administered simultaneously with aureomycin. Fourteen other cases were subjected to the same regimen except that a 250 mg aureomycin suppository was employed which contained 280 mg of mixed paraben esters. In 19 cases, two 250 mg gelatin capsules of aureomycin were inserted intravaginally each day for a week. Aureomycin alone was employed in 10 patients, while in 9 it was administered concurrently with Propion Gel. Finally, 10 patients were subjected to the following regimen: 160 mg of methyl paraben and 40 mg of propyl paraben, in the form of a capsule, were inserted intravaginally each day for a week. Using a Holmes insufflator, powder consisting of 1 g of aureomycin in 3.95 g of talc was sprayed evenly over the cervix, vagina, introitus and vulva. In 10 cases this powder contained no paraben, in 14 cases it contained 1% paraben (.8% methyl paraben and .2% propyl paraben) and in 29 cases it contained 10% paraben (8% methyl paraben and 2% propyl paraben). To summarize: 109 patients were observed; 27 received aureomycin alone, 57 aureomycin containing paraben, 15 aureomycin with Propion Gel and 10 paraben alone. Results are represented in Table I.

Rectal studies. Nineteen patients were included in this study. Each patient was proctoscoped on the first, third and fifth day. At each proctoscopic examination the mucosa was observed, cultures for yeast were obtained and medication was inserted. On the second and fourth days, medication was inserted digitally. In 4 cases, two 250 mg gelatin capsules of aureomycin without paraben were administered each day. Five patients were subjected to the same regimen except that each 250 mg capsule of aureomycin contained 142 mg of methyl paraben and 35.5 mg of propyl paraben. A 250 mg wax suppository of aureomycin was employed in 5 instances; while 5 additional patients were treated in exactly the same manner except that each aureomycin suppository contained 224 mg of methyl paraben, and 56 mg of propyl paraben. Therefore, in this phase of the investigation 9 received aureomycin alone and 10 aureomycin containing paraben. The results are summarized in Table I.

Discussion. In vitro studies using 5 strains

of yeast isolated from patients complaining of moniliasis following antibiotic therapy established a definite inhibitory effect by the methyl and propyl esters of paraben. Oral, vaginal, and rectal studies involving 186 patients demonstrated that the addition of these agents to aureomycin was of value in preventing the development of moniliasis. This was especially obvious in the oral studies. When aureomycin alone was administered, 63% of those patients whose stools were initially free of *C. albicans* subsequently revealed these organisms. In contrast, when aureomycin containing paraben was employed, only 13% disclosed yeast. While the number of patients involved in the rectal studies was limited, results were consistent with those observed in the oral and vaginal cases. With 1 exception, *C. albicans* infections became more severe with aureomycin therapy; however, no alteration was observed when aureomycin containing paraben was administered. The combination of aureomycin and paraben caused the disappearance of *C. albicans* in only 2 cases. In 3 patients having monilial vaginitis the intravaginal insertion of 200 mg of paraben daily for 3 weeks resulted in an amelioration of symptoms but did not eradicate the yeast. It appears from these studies that methyl and propyl paraben exert only a weak or moderate inhibitory action on *C. albicans in vitro* and *in vivo* but are capable of preventing its overgrowth during aureomycin treatment.

Little variation was noted in the occurrence of nausea, vomiting and diarrhea in patients receiving aureomycin alone and in those given aureomycin containing paraben esters. No toxic effects to the esters of paraben were observed.

Summary. (1) *In vitro* sensitivity studies established a definite inhibitory effect by methyl and propyl paraben (para-hydroxybenzoic acid) on 5 strains of yeast. (2) Methyl and propyl paraben are essentially non-toxic when administered orally, vaginally or rectally. (3) Methyl and propyl paraben are of value in preventing the overgrowth of *C. albicans* associated with aureomycin therapy.

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Comparison of Normal Blood Picture of Young Adults from 18 Inbred Strains of Mice. (19210)

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In this study of the formed elements of the blood of normal mice, a total of 18 inbred lines were investigated, including sublines of 10 major strains, a large proportion of those widely used in biological research(1-3). All are maintained together in the "inbred nucleus" of the Jackson Laboratory, where certain of the lines (marked with †) form the background of all mice sold to other institutions. Experimental mice were virgins, 2 to 3

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TABLE I. Mean Values for Erythrocyte Characteristics of Each Strain Tested. All means are based on 10 animals, except those marked (*) based on 20 animals (see text). Those strains marked (†) are major strains available to other laboratories.

Strain	RBC/mm³ Mean 10⁶	S.E.	Haematocrit Mean	S.E.	Mean cell vol, mu³ Mean	S.E.	Haemoglobin, g/100 cc blood Mean	S.E.	Haemoglobin, g/cc cells	Reticulocyte, %
A/Jax†	9.42	± .28	42.5	± .4	45.1	± 1.4	12.9	± .2	.30	3.5
A/HeJax†	9.48	± .18	42.5	± .5	44.8	± 1	12.7	± .2	.30	2.9
AKR/Jax†	9.38	± .24	45.6	± 1	48.5	± 1.6	13.9	± .2	.30	2.3
BALB/cAnJax	10.14	± .15	46.5	± .8	45.9	± 1.1	14.5	± .2	.31	3.3
BALB/cJax†	10.51	± .16	48	± .7	45.7	± 1	15	± .2	.31	2.9
CBA/Jax	10.04	± .27	45	± 1.3	44.8	± 1.8	13.5	± .2	.31	2.6
C3H/Jax†	8.79	± .24	39.5	± .7	44.9	± 1.4	12.2	± .4	.31	2.8
C3H/SeJax	9.63	± .26	43	± 1	44.7	± 1.6	13.2	± .3	.30	2.2
C57BL/6pJax	9.70	± .15	43.4	± .8	44.7	± 1	13	± .3	.30	2.5
C57BL/6Jax†	9.66	± .09	44	± .4	45.5	± .6	13.3	± .2	.30	2.6
C57BR/cdJax†	10.54	± .17	50	± .5	47.4	± .9	14.6	± .2	.29	2.4
C57L/HeJax†	9.82*	± .20	50.6*	± .4	51.5*	± 1.1	14.9	± .2	.29	2.6
DBA/1Jax†	10.52*	± .27	43.8*	± .6	41.6*	± 1.2	13.2	± .2	.30	1.5
DBA/WaJax	9.93	± .27	43	± .6	43.3	± 1.1	12.5	± .2	.29	2.6
DBA/2Jax†	10.30	± .25	42.6	± .5	41.4	± 1.1	12.7	± .1	.30	3.1
I/Jax	10.27	± .27	46.8	± .7	45.6	± 1.5	13.5	± .1	.29	2.4
RIII/Jax	9.63	± .25	44.5	± .6	46.2	± 1.3	13.7	± .2	.31	2.8
ST/Jax	9.88*	± .19	44.1*	± 1.1	44.6*	± 1.4	12.1	± .2	.31	2.1
Mean	9.92	± .07			45.3	± 1.3	13.4		.30	2.6

months old. Preliminary experiments had indicated that before this age the rapid growth of the animal and correlated changes in blood counts made determinations too variable. Five females and 5 males of each strain were used for each type of determination. It should be stressed that in no case has there been any direct selection by any investigator toward high or low levels of any type of blood cell (with the possible exception of high leukocyte counts in older animals of leukemic strains). Whatever values now characterize these strains have been attained either by natural selection or by chance genetic fixation. Other experiments of this type, studying the blood of certain inbred strains of mice, have been reported in the literature(3-14) but the breadth and lack of selection in this survey should make its results approach more closely a complete characterization of the normal level of formed elements of the blood of the young adult house mouse as a species than has previously been possible.

Technical methods. Standard methods were used for erythrocyte count, hematocrit determination, hemoglobin content, reticulocyte percentage, total leukocyte count and differential count. Special adaptations for the small samples available from single mice were the use of the Van-Allen hematocrit tube and the cyanomethemoglobin micromethod of Turner(15) for hemoglobin content. 1.3% sodium oxalate is isotonic for mice. The hemoglobin results were checked by duplicate determinations on samples pooled from several mice by the Turner method and the Sendroy(16) modification of the Van-Slyke-Neill(17) oxygen capacity method. Where two independent samples were taken from the same animals, accuracy of the results can be judged from the ratio of mean difference between samples to the grand mean for all animals combined (RBC/mm³, 5%; Ht, 2.3%, Hb, 2.8%, WBC/mm³, 13%). For all determinations tail blood was used, obtained after heating the tail in hot water. For erythrocyte counts, hematocrit and hemoglobin determinations, and total leukocyte counts, nembutal anesthesia was used since preliminary experiments gave lower counts and lower relative variability in animals so treated. In determinations of reticulocyte percentage, 500 cells were counted in smears prepared with alcohol solution of brilliant cresyl blue, counterstained with Wright's. A single differential leukocyte count from each animal (smear stained with Wright's) included 300 cells scattered at random over the slide. Although cells were re-

TABLE II. Mean Values for Leukocyte Characteristics of Each Strain Tested (Total No. and Proportion of Granulocytes). All means are based on 10 animals.

Strain	Total leukocytes/mm³ Mean 10^3	S.E.	% granulocytes ♀♀ Mean	S.E.	S.E./mean	♂♂ Mean	S.E.	S.E./mean
A/Jax†	8.71	± 1.37	10.7	± 1.1	.10	14.5	± 5	.35
A/HeJax†	6.14	± .68	19.8	± 2.3	.12	37.2	± 7.5	.20
AKR/Jax†	6.80	± .65	21.6	± 4.5	.21	23.8	± 5.2	.22
BALB/cAnJax	8.70	± 1.03	15.4	± 2.3	.15	29.6	± 5	.17
BALB/cJax†	8.54	± 1.04	14.9	± 2.1	.14	15.3	± 3.8	.25
CBA/Jax	6.44	± .33	26.8	± 4.5	.17	24.2	± 2.5	.10
C3H/Jax†	7.34	± .80	21.4	± 4.3	.20	27.5	± 5.1	.19
C3H/ScJax	5.07	± .30	19	± 3.9	.21	22	± 2.2	.10
C57BL/6pJax	10.61	± .64	12.1	± 3.8	.31	15.3	± 5.1	.33
C57BL/6Jax†	11.43	± 1.04	8.2	± 1.2	.15	10.4	± 3.5	.34
C57BR/cdJax†	9.43	± .78	10.6	± 1.1	.10	11	± 2.4	.22
C57L/HeJax†	10.82	± 1.13	8.5	± 1.3	.15	6.7	± 1.4	.21
DBA/1Jax†	8.60	± 1.60	16.5	± 1.5	.09	20.4	± 4.2	.21
DBA/WaJax	8.32	± .96	19.6	± .9	.05	17.2	± 1.5	.09
DBA/2Jax†	9.28	± .29	16.7	± 2.3	.14	18.1	± 1.3	.07
I/Jax	11.62	± 1.41	13.2	± 3.2	.24	18.1	± 2.9	.16
RIII/Jax	5.87	± .64	26.2	± 2.8	.11	18.6	± 3.7	.20
ST/Jax	7.74	± 1.23	14.9	± 2.4	.16	19.4	± 3.7	.19
Grand mean	8.41	± .26	16.5			19.4		
Mean, sexes combined				18				

† Major strains available to other laboratories.

TABLE III. Results of Analyses of Variance for Each of the Formed Element Determinations Tested.

Measurement	Source of variation	df	Mean square	F value	Level of significance, %
Erythrocyte No.	Total	179			
	Lines	17	5.37	4.93	.1
	Sex	1	0 –	–	—
	Line × sex	17	1.77	1.62	—
	Error	144	1.09		
Haematocrit determination	Total	179			
	Lines	17	201.50	22.74	.1
	Sex	1	6.95	.78	—
	Line × sex	17	16.72	1.88	5
	Error	144	8.86		
Total leukocytes	Total	179			
	Lines	17	75.11	3.80	.2
	Sex	1	11.77	.60	—
	Line × sex	17	14.56	.74	—
	Error	144	19.76		
% granulocytes	Total	179			
	Lines	17	338.9	6.99	.1
	Sex	1	382.5	7.89	1
	Line × sex	17	82.8	1.71	5
	Error	144	48.5		

corded as monocytes, lymphocytes, and neutrophilic or eosinophilic granulocytes for statistical purposes, only the two most distinct groups, granulocytes (almost entirely neutrophils) and agranulocytes (almost entirely lymphocytes) were classified. Smudged cells were omitted from the total, which could possibly lead to distortion of results. It is felt that the erythrocyte counts and hematocrit and hemoglobin determinations are sufficiently reliable to be accepted as standards for these strains and ages. The other determinations, due either to lack of precision in method or known variability with the state of the animal,

are useful only for determining relative status of strains and for approximating their absolute level.

Statistical methods. The mean and standard error was determined for each strain for each type of measurement, but in general the differences betwen strains are small enough, and there is sufficient overlap among the measurements, so that significant strain differences are not apparent by this method. However, using analysis of variance and comparing the proportion of the total variance attributable to the particular factors which can be separated out in this experiment (strain, sex, and factors involving an interaction of strain and sex) with the variance due to unanalyzed "errors" (experimental errors in method or sampling; environmental differences such as nutrition, temperature, exposure to pathogenic organisms, time of day, season of year, stress, etc.) it is possible to determine definitely whether or not the isolated factors contribute significantly to the total variation. In certain cases, Chi-square tests for non-random distributions were also used. Neither of these methods tells what the genetic factors are, nor in which strains they reside, but proves their reality.

Results. Tables I and II summarize the results by strain and Table III indicates the significance of contributions of tested sources to total variations observed for each variation. Genetic differences among the strains contributed very significantly to variations in all tested determinations. Sex, independent of strain, contributed significantly to only one determination, percent granulocytes. In addition, there is a possibility, significant at the 5% level only, that the strains differ in their tendency to favor expression of sex differences in hematocrit value and percent granulocytes. Since hemoglobin values correlated completely with hematocrit determinations on the same animal (Table I) no strain differences were found in hemoglobin concentration within cells, and conclusions for total hemoglobin content coincide with those for hematocrit level. Fig. 1 indicates that in the great majority of strains, variations in hematocrit mean can be explained by variations in cell number, as the ratio of Ht mean to RB mean falls

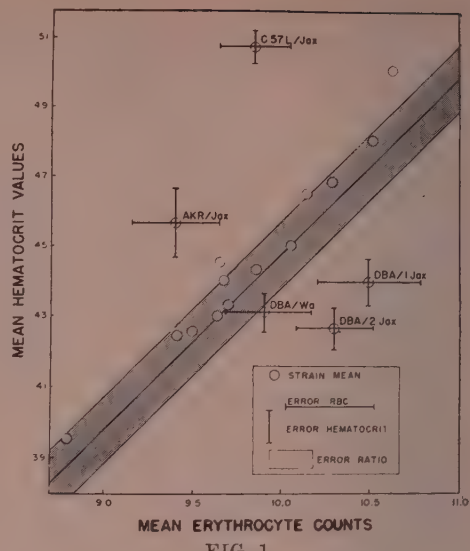

FIG. 1.

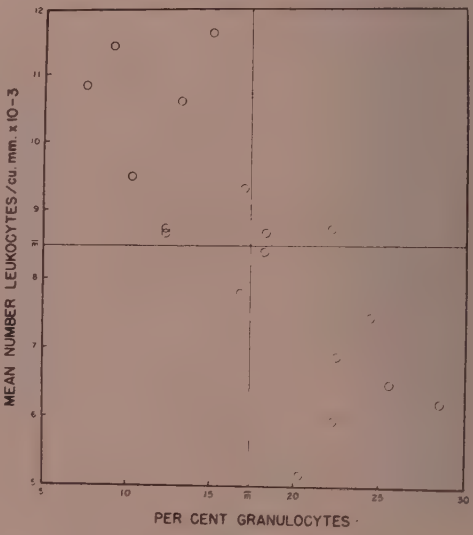

FIG. 2.

within the range of error of a straight line whose slope, 45.3 ± 1.3, is the mean cell volume of all strains combined. In a few strains (DBA/1Jax, C57L/HeJax, and ST/Jax) the recorded values are based on 20 rather than 10 individuals to test more critically for differences in cell size. One strain only, C57L/HeJax, showed a mean cell volume (51.5) significantly higher than that for all strains combined. The narrow low range of reticulo-

cyte percentage makes statistical analysis by strains impractical, so this measurement is given simply to indicate the typical level in 2-3 month old mice. Fig. 2 shows an inverse relationship between mean granulocyte percentage of a strain and the total leukocyte count for the same strain, indicating great strain variability in absolute number of lymphocytes combined with small or insignificant strain difference in absolute granulocyte number. Another interesting finding is a highly significant difference in proportion of granulocytes between 2 sublines of a single major strain, separated in 1927 after the lines had been more than 20 generations inbred. The A/HeJax had the highest percentage of any tested strain, while A/Jax is considerably below the grand mean. For two determinations (RBC/mm^3, A-strain subline difference in % granulocytes) Chi-square tests for possible significant differences due to portions of the year or related factors were negative.

Discussion. A study of the formed elements of the blood in young adults of 18 different inbred strains has resulted in a characterization of the normal level for the laboratory mouse for each type of determination. It was possible by statistical methods, particularly the analysis of variance, to demonstrate that a very significant contribution to the observed variations in erythrocyte number, mean cell volume (one strain only), total leukocyte count, and proportion of granulocytes to agranulocytes came from genetic differences among the inbred strains used. Related inbred strains in many cases seem to have been fixed for some of the same genes during their common history. The similarity of total and differential leukocyte counts of the entire C57 group offers a good example of this phenomenon. On the other hand, related strains or sublines occasionally differ by genes unfixed at the time of separation or by new mutation. In one case (the differential counts of A/Jax and A/HeJax) there is a highly significant difference between sublines separated after more than 20 common generations of inbreeding. Few of the differences in this data are due to sex alone. The animals are sexually mature, and would have had litters if mated, but hormones or other factors associated with sex have a strong effect on only one of the determinations, the proportion of granulocytes in the differential (Tables II and III). However, sex may also interact with genetic difference among strains to alter granulocyte percentage and hematocrit level (Table III). The inverse correlation between total leukocyte count and proportion of granulocytes (Fig. 2) suggests that differences in genetic factors affecting production or release of lymphocytes are more prevalent or more effective than are genes affecting granulocytes.

In each of these inbred strains, the observed values must fall within the limits of normality for the line to persist. The inbreeding process would eliminate quickly any genes severely deleterious to individuals before the height of the reproductive period. The results indicate that the strains differ from each other in genes with beneficial, neutral, or only slightly deleterious effects. Inbred strains may differ from each other in patterns of physiological balance, and blood characteristics may be correlated with differences in other characters. Physiological attributes to which erythrocyte level might be related are degree of bodily activity, basal metabolism, or, following the suggestion of Strong(4), tendency to develop mammary cancer. Hemoglobin content shows no clear-cut relation to activity level, as judged by this laboratory's investigators handling the mice. The most active DBA group ranged from 12.5 to 13.2 mean g Hb/100 cc, and the least active C3H group ranged from 12.2-13.2 mean g Hb/100 cc blood. BALB/c and C57BL/6 strains, with intermediate activity range from 14.5-15.0 g Hb/100 cc blood. No data are available on basal metabolism. Strong's hypothesis that age drop in hemoglobin of females with tendency to develop mammary cancer cannot be tested here. At the tested ages of 2-3 months, the mean hemoglobin content of mammary tumor strains (A/HeJax, C3H, CBA, all DBAs, RIII, and ST) ranged from 12.1 to 13.7 g Hb/100 cc blood, while that of tumor-free strains (BALB/c, all C57s, and I) ranged from 13.0 to 15.0 g Hb/100 cc blood. It is doubtful but not impossible that this slight range difference has meaning in relation to prospective tumor incidence. Many environ-

mental factors have been shown to influence total leukocyte and differential counts(18,19), but the only genetic ones for which we have definite knowledge of these particular strains are antibody-building response and tendency to develop leukemia. The antibody production of five of these strains (DBA/1, BALB/c, C3H, C57BL/6, and A) has been tested against pneumococcus polysaccharide and egg albumin(20) with significant strain differences completely unrelated to total or differential leukocyte count. Mice which later develop lymphatic leukemia might tend to have high lymphocyte counts. However, AK/RJax, in which 80% of the mice die with lymphatic leukemia before one year, gave a low mean of 6.80 leukocytes/mm³. Older mice of DBA/2Jax and older males of C3H strains occasionally develop leukemia, and these also have low leukocyte counts at 2-3 months. It has thus not been shown that an observed strain difference in formed elements is one facet of a difference in a particular known pattern of physiological balance.

Another explanation for the observed strain differences might be chance fixation of a particular genotype. Environmental variations may have been so great as to prevent effective natural selection in each strain of the same best type. Or it may equally well be that there actually is no selective advantage within the total observed range. If this is so, this survey should not only prove the existence in normal mice of genes affecting the formed elements of the blood, and their differential distribution among the tested inbred strains, but should come close to establishing the range of values normal for the species as a whole.

Summary. (1) Erythrocyte count, hematocrit determination, hemoglobin content, reticulocyte percentage, total leukocyte count, and proportion of granulocytes were determined in the blood of 2 to 3 month old virgin males and females of 18 different inbred strains. The means and standard errors for each determination are given for each strain. (2) Although there was considerable overlap among the strains, it was possible by analysis of variance to show that there were significant genetic differences among the strains in factors affecting erythrocyte number (as shown both in count and hematocrit), and mean erythrocyte volume (one case only), total leukocyte count, and proportion of granulocytes. (3) Mean total leukocyte count for a strain was negatively correlated with proportion of granulocytes. (4) In this data, the observed strain differences do not appear to be related to differences in other known physiological characteristics.

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Anastomosis of Auricular Appendages in Dogs.* (19211)

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It has been suggested that relief of pulmonary edema in certain instances of mitral stenosis may be accomplished by the establishment of an artificial Lutembacher's syndrome. This suggestion would entail the production of an interatrial septal defect or its equivalent to provide a safety valve for the left auricle during periods of especially high pressure, so that congestion of the lungs would be prevented(1,2). In addition to the use of an interatrial defect, or its equivalent in the above mentioned instance, it has been suggested that its presence in certain congenital cardiac malformations is desirable for prolongation of life(3). The production of this defect, by direct attack on the interatrial septum itself, has met with more failure than success. The reasons for this difficulty have been due to the following: 1. Blind approach necessary. 2. Tendency for defects to close spontaneously.

It occurred to us that a form of interatrial septal defect might be produced that would avoid both of the difficulties mentioned, namely, the direct anastomosis of the auricular appendages. The purpose of this presentation is to test this latter thesis experimentally.

Methods. These experiments were carried out in dogs in 3 series. In each series, the dogs were anesthetized with nembutal and with the use of an intratracheal tube and positive pressure, inflation of the lungs was maintained. Under aseptic conditions the right chest was entered through the fourth interspace. The right lung was carefully packed out of the field and the pericardium entered. The right auricular appendage was then delivered and a Blalock vascular clamp placed around its base. The left auricular appendage was then also delivered by slightly rotating the heart. A similar clamp was then placed about its base. One of the following 3 procedures was then undertaken:

Series I. *Direct anastomosis of auricular appendages.* The ends of the appendages were resected. Traction was then applied to both clamps and the two open ends of these organs approximated. An everting type of anastomosis with 6-0 arterial silk was then done. Interruption of the continuous suture was accomplished at 3 places, so a purse-stringing effect would be avoided. The clamps were then removed, and only a fair flow of blood was felt going through the anastomosis from left to right. A thrill could be palpated at the site of anastomosis. The chest was then closed in the usual manner. Series II. *Vein or arterial graft between auricular appendages.* Segments of preserved inferior vena cava vein or aorta(4) were inserted between the appendages by means of an everting mattress suture, or by the non-suture technic of Blakemore(5). When the latter technic was used, the necessary cuffs were constructed of finely polished lucite. Series III. *Lucite prosthesis between the two auricular appendages.* Finely polished lucite prostheses were inserted between the two appendages. These tubes were so constructed so as to have a small flange at each end, around which the appendage was tied with a single suture. The sizes of prostheses used were a 3 cm length with 3 mm internal bore, and a 2 cm length with a 2 mm bore. After these tubes were tied in place to both appendages, the pericardium was then sutured together above the area. When the Blalock clamps were removed, arterial blood could be seen coursing through.

Results. Series I. *Direct anastomosis.* Four dogs were used in this series. The animals were sacrificed after a period of 2 months. Post-mortem examination revealed that all but one of the anastomoses had developed stricture and were therefore occluded. In the one animal with a patent opening, the sutures could be seen from within, covered with a lining of epithelium. It appeared as if the

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anastomosis was not carrying a good shunt of blood, but some apparently was coursing through. A point of note in all the animals was that the auricular appendages seemed to be under tension and thus the soft wall of anastomotic area was collapsed.

Series II. *Vessel graft between auricular appendages.* Five dogs were used in this series. In 2 of the animals the non-suture technic was used, and in the remaining 3, suture method was used. After a period of from 1 to 2 months the animals were sacrificed. All of the communications were thrombosed. In the non-suture method, it was noted that though the vessel grafts were intact grossly, a stricture-like effect had occurred at each end of the graft on the adjacent atrial wall. Thrombus blocked the tiny openings that remained. Whether the stricture occurred as a result of an inflammatory reaction to the lucite "cuffs," or of the tendency for the atrial wall to undergo stricture because of its muscular thickness, is not known at this time.

Series III. *Lucite prosthesis.* Four dogs were used in this series. After a period of from 1 to 2 months the animals were sacrificed. Thrombosis had occurred in each of the communications. It was noted that though thrombus was present at each auricular end, the prostheses themselves, contained material (serum?) of a liquid character. The tubes and the appendages were firmly connected and could not be separated except by sharp dissection. A thin layer of smooth, fibrous tissue lay immediately adjacent to each tube.

Comment. It is apparent from these experiments that though the possibility of the creation of an interatrial communication by the anastomosis of auricular appendage exists, the problem of thrombosis is a major one. However, it must be mentioned that the above experiments were carried out in normal dogs, and in these animals the pressure differential between the left atrium and the right atrium is very small (2-4 mm Hg). Such a small differential is not conducive to a good flow of blood, which in turn, is necessary in vascular anastomoses. It is possible that in mitral stenosis, where a differential of 25-40 mm of Hg is obtained, the anastomoses will remain open. Intubation of the thoracic aorta with lucite has been accomplished successfully in the dog(6) and the success of this would seem to be due to the excellent flow of blood in this vessel, thus preventing stasis and thrombosis.

Summary and conclusions. (1) Methods of producing interatrial communications by anastomosis of the auricular appendages are presented. (2) These methods are technically feasible. (3) Thrombosis has prevented the functioning of these communications for as long as 1 or 2 months.

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Biotin Nutriture and the Enzymatic Incorporation of $NaHC^{14}O_3$ into Acetoacetate. (19212)

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In previous studies it was demonstrated that the carbon of C^{14} bicarbonate can be incorporated into the carboxyl group of enzymatically generated acetoacetate by various animal tissue preparations(1-3). It was also shown(1) that there was less incorporation of C^{14} into acetoacetate by rat liver homogenate systems from biotin deficient animals than from those which received this vitamin in the diet or by injection. However, since acetoacetate production by the homogenates was not determined in the earlier work, it could not be decided whether the depressed C^{14} incorporation by biotin deficient tissues could be ascribed to diminished acetoacetate formation or to a specific decrease of the incorporation of C^{14} from bicarbonate.

Methods. Weanling Sprague-Dawley rats were grown for 10 weeks on a purified diet containing raw egg white without added biotin (1,4). The animals fed egg white developed typical symptoms of biotin avitaminosis. Some of these deficient animals were then injected with 50 γ of biotin daily for 2 days and their food intake adjusted to that of non-treated rats of the same body weight. The livers of such pairs of animals were used for the comparison of enzymatic activity. The reaction mixture(5), methods of incubation, and the procedures used for the degradation of acetoacetate and counting of radioactivity were identical with those previously described(1,3). The exact amounts of carrier and metabolically produced acetoacetate were determined by the method of Greenberg and Lester(6). The values for the "corrected" specific radioactivity of the carboxyl group of acetoacetate were calculated in the same manner as previously indicated(3). The results reported are based on equivalent dry weights of homogenates.

Results. The state of biotin nutrition of the rats did not significantly affect the quantity of acetoacetate produced by the liver homogenates with either pyruvate, caprylate or

TABLE I. Effect of Biotin Deficiency on Incorporation of C^{14} from $NaHC^{14}O_3$ into the Carboxyl Group of Acetoacetate.

Substrate	Biotin treatment	Acetoacetate, metabolically formed, μM/ml	Radioactivity ("corrected")* of metabolically formed acetoacetate, cts/min/μM
Pyruvate	—	.34	0
	+	.39	5.04
Caprylate	—	1.30	.48
	+	1.38	49.2
Caproate	—	.73	4.68
	+	.78	78.6
Isovalerate	—	0	5.82†
	+	0	73.8 †

All substrates added to .0012 M. Incubated at 37° in air for 30 min. 4.2×10^4 cts/min $NaHC^{14}O_3$ added per flask. Error of count is within $\pm$ 2%.

* c.p.m. per μM "corrected" = [μM acetoacetate $\times$ c.p.m./μM (substrate present) — μM acetoacetate $\times$ c.p.m./μM acetoacetate (control)]/[μM acetoacetate (substrate present) —μM acetoacetate (control)]. Detailed calculations are described in Reference 3.

† Radioactivity in presence of acetoacetate carrier. Corrected for incorporation of C^{14} in absence of isovalerate.

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caproate as a substrate. At the same time there was a 15-100 fold greater incorporation of C^{14} bicarbonate into the carboxyl group of acetoacetate produced by homogenates of livers from biotin repleted rats than by the deficient ones.

With the homogenate systems employed here, isovalerate leads to a marked increase in the radioactivity of the carboxyl group of acetoacetate, though no significant net formation of ketone bodies can be detected(3). However, biotin treatment of the deficient rats resulted in a 12-fold increase of C^{14} incorporation into acetoacetate derived from isovalerate.

The decreased *incorporation* of C^{14} from bicarbonate into the carboxyl group of acetoacetate by rat liver homogenates from biotin deficient animals as compared to those from the repleted ones seems to be the result of an effect on the bicarbonate incorporating mechanism, since the *amount* of this keto acid produced by the liver homogenates from a number of substrates was not influenced.

Summary. The quantity of acetoacetic acid produced metabolically from pyruvate, caprylate, and caproate by rat liver homogenate systems is not affected by the state of biotin nutrition of the animals. However, biotin treatment of deficient animals increased by 10-100 fold the incorporation of C^{14} bicarbonate into the carboxyl group of acetoacetate metabolically formed from pyruvate, caproate, caprylate or isovalerate.

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Blood Glucose Levels in Alloxan-Diabetic Rats under Combined Insulin and Ergot Alkaloid Therapy.*† (19213)

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Dubois, Herrmann, and Erway(1) have reported that the hyperglycemia produced by the rodenticide, alpha-napthylthiourea (ANTU), is conteracted by ergotamine, which is a natural alkaloid of ergot, as well as by insulin. They accounted for this parallelism through assumption that there are antagonistic actions of both ergotamine and insulin toward the glycogenolytic action of epinephrine, the production of which is stimulated by the rodenticide. Stoll and Hofmann(2) have been responsible for the production of dihydrogenated derivatives of the ergotoxine group of ergot alkaloids. It has been shown by Rothlin(3) that the hyperglycemia produced in animals through administration of epinephrine is inhibited by the hydrogenated ergot alkaloids. Freis, Stanton, and Wilkins(4) have demonstrated adrenolytic activity of dihydroergocornine (DHO 180) and dihydroergokryptine (DHK 135) in humans and have also shown, under proper experimental conditions, that the pressor response, tachycardia, and mydriasis which normally follow epinephrine administration are reduced by these preparations. Schneider(5) found that the period of hypoglycemia in humans, produced by the intravenous injection of small doses of insulin, was not lengthened by administration of dihydroergocristine, dihydroergocornine, or

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dihydroergokryptine. It has been shown by Nitsch(6) that Hydergine (CCK 179) which is a mixture of dihydroergocornine, dihydroergocristine, and dihydroergokryptine, injected before administration of insulin, served to reduce oscillations of blood glucose to a considerable degree when such oscillations were fairly marked but that the preparation had little or no tendency to potentiate the action of insulin in those cases with only slight blood glucose oscillations.

The present investigation was originally designed to determine whether the insulin regulation of blood glucose levels would be interfered with by the administration of the dihydrogenated derivatives of ergot. The effects upon the blood glucose levels of insulin-treated alloxan-diabetic rats resulting from administration of ergotamine tartrate, dihydroergocornine, and Hydergine are compared.

Materials and methods. Albino rats of the Sprague-Dawley strain and of both sexes were used in this investigation. All animals were between 100 and 150 days of age and all were fed a stock diet commercially manufactured and containing the usual proportions of protein, fat and carbohydrate; drinking water was supplied *ad libitum.* The induction of alloxan diabetes was accomplished through intraperitoneal injection of alloxan monohydrate (approximately 150 mg per kg of body weight) dissolved in distilled water as described by Gomori and Goldner(7). It was considered that a diabetic state existed when the blood glucose level reached or exceeded 200 mg %. As has been previously shown by the report of Sinden and Longwell(8), it was found that the amount of protamine zinc insulin necessary to maintain satisfactory blood glucose levels was more or less arbitrary. In general, the following amounts were found to be fairly satisfactory: 7 units protamine zinc insulin per 24 hours if the blood glucose in mg % was over 500; 5 units protamine zinc insulin per 24 hours if the blood glucose level in mg % ranged between 250 and 500: 3 units protamine zinc insulin per 24 hours if the blood glucose level in mg % ranged between 200 and 250. Insulin was injected subcutaneously at 24-hour intervals. Determinations of blood glucose levels were accomplished through removal of blood samples from the tips of the tails of the rats with heparin being used as an anti-coagulant. Blood glucose determinations were made according to the method of Folin and Wu(9) as modified by Fiorentino and Giannettasio(10). Blood glucose levels were determined in normal and alloxan-diabetic rats immediately prior to insulin administration and at intervals of 1, 3, 7, and 10 hours after it had been given. Accordingly, the first blood sample was obtained at 10:00 a.m., insulin was given immediately thereafter, and subsequent determinations of blood glucose levels were made at 11:00 a.m., 1:00 p.m., 5:00 p.m., and 8:00 p.m. When insulin administration was omitted blood glucose determinations were carried out at the same hours in order to provide accurate comparisons of blood glucose levels in insulin-controlled and uncontrolled alloxan-diabetes. To study the effects of combinations of protamine zinc insulin with Hydergine, dihydroergocornine or ergotamine tartrate, the ergot alkaloids were injected intraperitoneally 2½ hours after a regular 24-hour subcutaneous injection of insulin in the amount of 4 mg per kg of body weight. The time interval of 2½ hours between injection of insulin and that of the alkaloids was selected because it was found, once insulin control of the blood glucose level in the alloxan-diabetic rat had been established, that the period during which the blood glucose level remained fairly constant was between the third and tenth hours following the injection of insulin. Blood glucose levels under combined therapy were determined at the same intervals and at the same times of day as in the normal and alloxan-diabetic animals.

Results. The blood glucose determinations for each series of animals are presented in Table I. That diabetes was present in the alloxan-treated animals is evident when parts a and b of Table I are compared. The effectiveness of insulin in controlling the blood glucose levels of alloxan-diabetic rats is readily apparent when one compares part c of Table I with part b. Examination of Table I, d

TABLE I. Blood Glucose Determinations in mg % in Normal, Diabetic, and Diabetic Rats Under Therapy.

	10 a.m.	11 a.m.	1 p.m.	5 p.m.	8 p.m.
a. Normal, untreated	108	120	128	112	120
	112	130	120	116	116
	116	136	130	116	116
	110	115	112	116	118
	114	120	125	118	112
Avg	112	124	123	116	116
b. Diabetic, untreated	225	245	240	700	240
	425	375	440	375	345
	570	570	570	550	475
	335	340	370	350	335
	420	450	430	430	450
Avg	395	396	410	481	369
c. " insulin treated	76	56	46	42	104
	144	128	84	52	136
	124	112	64	124	116
	68	64	52	66	64
	156	76	84	30	64
	120	64	40	40	52
Avg	115	83	62	59	89
d. " " " + ergotamine	398	276	42	42	68
	140	120	112	52	116
	284	354	60	42	48
	372	364	184	90	34
	112	112	34	68	34
	316	316	140	120	76
	140	140	60	42	40
Avg	251	240	90	65	59
e. " " " + CCK 179	135	80	45	60	50
	140	325	45	45	50
	350	60	40	60	70
	80	60	60	50	55
	480	440	55	85	130
Avg	237	193	49	60	71
f. " " " + DHO 180	208	212	36	46	58
	120	64	42	52	68
	280	152	112	64	76
	60	58	52	38	64
	60	126	52	46	60
	48	64	36	42	58
	144	46	46	46	58
	360	152	60	108	64
	152	112	60	84	94
	184	94	52	84	94
Avg	162	108	55	61	69

indicates that the combination of ergotamine and insulin resulted in a more prolonged and somewhat more pronounced depression of blood glucose levels than was produced by insulin alone. The administration of Hydergine (CCK 179) or dihydroergocornine (DHO 180) with insulin, as indicated in parts e and f of Table I, did not appreciably change the blood glucose levels from those resulting from insulin alone as observed over a 10-hour period; as with insulin alone the hypoglycemia was beginning to moderate at the end of 10 hours and this compared with a downward trend at the same interval in the animals given insulin and ergotamine tartrate.

The average mg % change in blood glucose levels between the third and tenth hours after insulin injection and after insulin injection followed in 2½ hours by injection of one of the alkaloids was calculated. In those diabetic rats treated with insulin the average blood glucose level increased an average of 28 mg % between the third and tenth hours after insulin injection. When ergotamine was combined

with insulin, the average blood glucose level dropped 31 mg % during the same interval. Combinations of Hydergine and dihydroergocornine with insulin were associated respectively with rises of 22 mg % and 8 mg % during the interval between the third and tenth hours after injection of insulin. When the "t" test of the difference between means of small samples was applied, the difference between insulin-treated and insulin-plus-ergotamine-treated animals showed a probability ("P") of 0.05. The probabilities when Hydergine or dihydroergocornine were administered in conjunction with insulin, were respectively 0.75 and 0.2. The probability values indicate that the effects upon blood glucose levels resulting from combined administration of ergotamine and insulin are statistically more significant than those produced by combined administration of Hydergine or dihydroergocornine and insulin.

Discussion. Since adequate control of blood glucose levels is essential during treatment of diabetic gangrene, diabetic ulcers, or other complications of diabetes, it is important that any therapeutic agents employed in conjunction with insulin do not alter appreciably its effect upon blood glucose. It appears that Hydergine and dihydroergocornine have relatively less marked effects on the blood glucose level when combined with insulin therapy than is the case when insulin and ergotamine tartrate are combined.

With regard to the use of Hydergine or dihydroergocornine in the treatment of the circulatory complications of diabetes, it has been demonstrated that they produce peripheral vasodilatation(11) which is usually beneficial in the treatment of such complications. Rothlin(12) has stated that the hydrogenated alkaloids constituting Hydergine are the only known substances known up to now which have, simultaneously, the properties of: (a) dilating the vessels and lowering arterial tension by manifest action upon the vasomotor center; (b) preventing, by inhibition of the pressosensory reflexes, a compensating increase in arterial tension; (c) assuring, due to their central bradycardic effects, an adequate cardiac output by adaptation of the heart to the increase in peripheral flow.

Tests(13) have shown that the average lethal dose of Hydergine is 100 to 1000 times as great as its effective dosage. It seems likely, on the basis of such tests, and on the basis of the observations presented herein that either Hydergine or dihydroergocornine may be used in the diabetic patient without significantly disturbing blood glucose levels as maintained by insulin therapy.

Summary. A series of alloxan-diabetic rats under insulin therapy was treated with ergotamine tartrate, Hydergine (CCK 179) and dihydroergocornine (DHO 180). The following results are recorded: Ergotamine tartrate lowered the average blood glucose value during a period of 3-10 hours after the regular 24-hour insulin injection; dihydrogenated ergot alkaloids, (dihydroergocornine or Hydergine, which is a mixture of dihydroergocornine, dihydroergokryptine and dihydroergocristine) on the other hand, seem to have little effect on the blood glucose level as regulated by insulin therapy.

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Effect of Total-Body X Irradiation on Metabolism of the Rat. (19214)

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Increased metabolism following X irradiation has been claimed on the basis of indirect evidence from the dog(1,2) and of basal oxygen consumption data from the rat(3). The present report indicates that total-body exposure of the rat to X rays does not affect metabolism except under special conditions.

Experimental. Male, Sprague-Dawley rats were subjected to single, total-body X irradiation (200 kv, 15 ma, 0.5-mm Cu and 3.0-mm Bakelite filters, 72.5 cm target distance, 0.9-mm Cu half-value layer and 15 to 20 r per minute dose rate). Six to eight rats were irradiated simultaneously, equal numbers of each experimental group of a study being included in an exposure. The animals were individually caged beginning one week prior to initiating experimental procedures. Drinking water was available at all times. Body weight was measured between 8:30 A.M. and 10:00 A.M. daily in the following groups of 17 rats each in 2 experiments: (1) complete deprivation of food, (2) 800 r and complete deprivation of food, (3) free access to food, and (4) 800 r and free access to food. Measurements were also made of the water content of the gastrocnemius muscles and of the whole carcasses of starved and starved irradiated (800 r) rats. Water content was determined by drying to constant weight in an oven at 102°C. In 3 experiments oxygen consumption of irradiated and control rats was measured in a multiple Regnault-Reiset apparatus (4) allowing the simultaneous, individual study of 6 animals for a 3 hour period. Prior to irradiation the oxygen consumption of the rats was measured daily for about a week. In each study there were 6 control and 6 irradiated rats. Three rats from each group were studied in the morning and 3 in the afternoon, an individual rat being measured during the same session each day. During all pre-irradiation periods, the rats were allowed access to food only during the first hour following their stay in the metabolism ap-

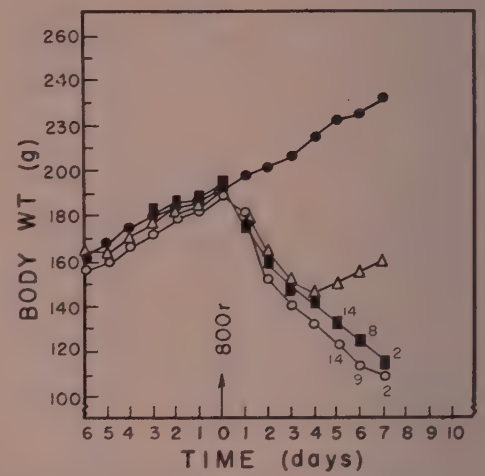

FIG. 1. Influence of total-body X irradiation upon body wt of starving rat. ● Fed, △ fed + 800 r, ■ starved, ○ starved + 800 r. Each point represents the mean of a group of 17 animals unless otherwise designated.

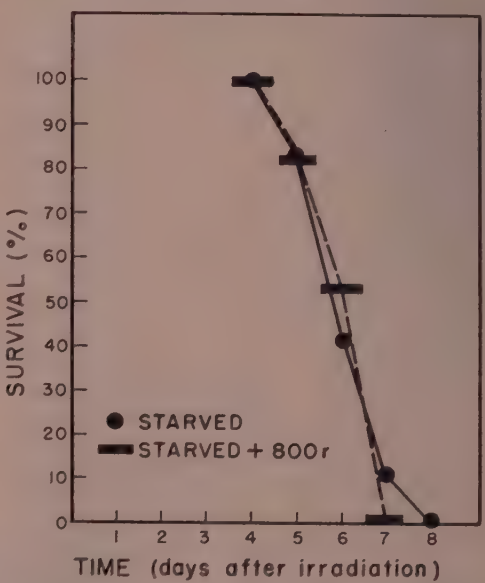

FIG. 2. Influence of total-body X irradiation upon survival time of starving rat. Each point represents the mean of a group of animals. Each group initially contained 17 rats.

TABLE I. Influence of Total Body X Irradiation Upon Water Content of the Rat.

Treatment group	Time after irradiation (days)	No. animals	Carcass water content				Gastrocnemius water content			
			Mean wet wt	Stand. error	Mean dry wt	Stand. error	Mean % water	Mean wet wt	Stand. error	Mean dry wt	Stand. error	Mean % water
Starved	1	7	191.9	±.5	56.8	±1.0	70.4	1.478	±.090	.380	±.028	74.4
" + 800 r		6	205.9	±3.5	60.7	±1	70.5	1.560	±.092	.394	±.025	74.8
"	2	8	184.6	±4.8	53.8	±1.8	70.6	1.235	±.035	.304	±.010	75.5
" + 800 r		9	187.9	±5.2	55.4	±1.4	70.3	1.342	±.054	.329	±.010	75.4
"	3	15	147.3	±3.6	42.3	±1	70.8	.977	±.040	.736	±.009	75.4
" + 800 r		10	133.3	±4	41.9	±1.6	68.3	.821	±.034	.701	±.008	75.6

paratus. Subsequently the experiments were as follows: (1) Exp. No. 1, 800 r and complete deprivation of food; (2) Exp. No. 2, 1000 r and complete deprivation of food, and (3) Exp. No 3, 800 r with access to food according to pre-irradiation pattern. Following irradiation, measurements of oxygen consumption were taken daily until death.

Results and discussion. The weight loss of starved rats was similar to that of irradiated, starved rats. Exposure to X radiation did not accelerate the rate of weight loss or hasten the death of rats that were completely deprived of food (Fig. 1 and 2). Neither did X irradiation influence the water content of the gastrocnemius muscle or of the entire carcass of the starved rat (Table I) indicating that water retention was not responsible for the failure to find an increased rate of weight loss in the starved-irradiated rat. France(5), found slight but statistically significant increases in the muscle water of the fed rat and in the total-body water of the fed mouse after 700 r. These increases might result from small differences in the nutritional status of his irradiated and control animals. Fig. 3 and an analysis of variance(6) show that: (1) irradiation did not increase the oxygen consumption of either the fed or the starved rats to values above the pre-irradiation control levels; (2) irradiated, starved animals had an overall trend toward a higher oxygen consumption than their non-irradiated starved controls; (3) rats with free access to food following exposure showed levels of oxygen consumption similar to those of the non-irradiated, fed controls, and (4) in agreement with previous studies(7,8) starvation was followed by a decrease in oxygen consumption.

The findings that the rate of weight loss of starving, irradiated rats and of starving controls is similar and that the basal oxygen consumption of fed, irradiated rats and of fed controls is similar strongly suggest that total-body X irradiation does not affect metabolism. Like oxygen consumption data from the fed, irradiated mouse(9), the observations(10,11) that tissue slices taken from irradiated animals show either no change or a decrease in oxygen consumption, and the report(9) that

FIG. 3. Influence of total-body X-irradiation upon basal oxygen consumption of the rat. Each point represents the mean of a group of 6 rats unless otherwise designated.

irradiated mice are less sensitive to progressive hypoxia lead to the same conclusion.

In the previously reported experiments which claimed an increased metabolism it was found that (1) dogs exposed to single, total-body X irradiation in the lethal dose range lost weight at greater rates than did pair-fed controls, and (2) following single, total-body X irradiation over the dosage range of 54 r to 972 r rats showed post-absorptive oxygen consumptions significantly in excess of their pre-irradiation control levels. The differences between these data, and our own seem more apparent than real. Thus the small number of dogs (3 control and 3 experimental) could readily have given misleading results, since there was considerable variability in the rates at which individual dogs lost weight when either partially or totally starved. The differences in oxygen consumption data can be explained by the possibility that in the study of Kirschner *et al.*(3), the post-irradiation measurements might have increased during the same period of time in the absence of irradiation. In our experiments the chances for such errors were obviated by the simultaneous study of non-irradiated, but otherwise similarly treated rats. Moreover, in the present experiments as well as in the recent report on the mouse(9) significant day to day variations in the controls were observed.

The overall trend toward a small but significant difference in oxygen consumption between irradiated, starved rats and non-irradiated, starved controls indicates that the metabolism of irradiated, starved animals is higher than that of controls. It is interesting to note that the greatest differences occurred during the time when widespread tissue regeneration becomes apparent in the fed rat (12). Should similar regeneration occur in the starved animal, it is conceivable that an increased oxygen consumption could be related to the processes involved in regeneration. The absence of significant differences in oxygen consumption during the first 2 or 3 days following irradiation in the fed groups and the small differences in the starved groups strongly suggest that cellular destruction is accompanied by little or no increase in metabolism. Failure to find indications of increased metabolism from the data on rates of weight loss in similarly treated rats may be explained on the basis that the differences in metabolism were too small to be reflected in body weight. Moreover, the data of Kirschner *et al.*(3) show a decided lack of parallelism between oxygen consumption and body weight changes.

Summary and conclusions. The resting oxygen consumptions of irradiated and control, fed animals are similar. The rate of weight loss of starving, irradiated rats is similar to that of starving controls. The water content of the whole carcass and of the gastrocnemius muscle of the starving rat is unchanged by irradiation. Oxygen consumption falls during starvation. The oxygen consumption of the starving, irradiated animal is higher than that of its simultaneously starved control. Apparently the metabolism of rats having free access to food is not affected by irradiation and the metabolism of the irradi-

ated, starved animals is higher than that of their non-irradiated controls.

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Nutrition of *Leptospira canicola.* II. A Chemically Defined Basal Medium Containing Purified Rabbit Serum Albumin.* (19215)

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It was shown by Greene *et al.*(1) that *Leptospira canicola* could be grown in a chemically-defined medium of amino acids, minerals, purines, pyrimidines and vitamins supplemented with dialyzed rabbit serum. Attempts to fractionate the dialysed serum by ammonium sulfate precipitation resulted in about 80% loss of the activity and the remaining activity was not concentrated in any fraction. In the present investigation an active fraction obtained by a modified procedure has been found to replace dialysed rabbit serum.

Experimental. The chemically-defined basal medium was prepared by introducing 0.5 ml each of solution A (36 mg % of each of the 19 amino acids employed previously)(1), solution B (vitamins, purines and pyrimidines in the concentrations given previously)(1) and solution S (autoclaved and filtered aqueous solution of the following salts at mg per cent concentrations given in the parentheses: Na_2HPO_4 (312), KH_2PO_4 (420), NaCl (1280), Na_2CO_3 (32), KCl (32), $CaCl_2$ (32)) into each 15 x 127 mm test tube. The tubes were autoclaved for 30 minutes at 15 lb pressure, 0.5 ml of solution G (36 mg % filter-sterilized aqueous solution of L-glutamine) and a selected volume of test material were added to each tube, each tube was inoculated with a culture of the test organism and each solution was diluted to 6 ml. The pH of the medium was 7.2-7.4. The transfer medium was prepared by adding 0.8 ml of filter-sterilized, dialysed rabbit serum‡ to each tube containing an autoclaved mixture of 0.5 ml of solution S, 3.96 mg of Witte's peptone and 4.3 ml of water. *Leptospira canicola*§ was transferred weekly by inoculating this medium with 0.4 ml of a 7-day culture of the spirochete and incubating the mixture for 7 days at 32°. The inoculum was prepared by centrifuging (2000 RPM for ½ hr) the 7-day growth, suspending the cells in a quantity of solution S sufficient to produce a turbidity of 30-40 de-

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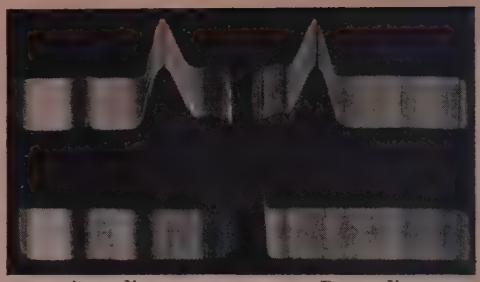
termined with a Klett-Summerson photoelectric colorimeter equipped with a No. 42 blue filter. 0.4 ml of this suspension was added to each tube in subsequent experiments. Photometric readings were made at the time of inoculation and after 7 days incubation at 32° to determine the effect of test materials. The whole rabbit serum was fractionated in preliminary experiments by mixing varying amounts of solid ammonium sulfate with 5.0 ml aliquots of the serum and allowing the mixtures to stand overnight in the refrigerator. The suspensions were centrifuged, the supernatant liquids were decanted and each precipitate was suspended in an ammonium sulfate solution of the same concentration as that employed to obtain the precipitate. Each suspension was centrifuged, the sediment was dissolved in 0.85% saline solution and the mixture was diluted to 4.0 ml. Each supernatant liquid was diluted to 4.0 ml. Each sample (suspension or supernatant liquid) was dialysed against 0.85% saline to remove excess ammonium sulfate, and each dialysed sample was diluted to 5.0 ml. Precipitation of globulins was prevented by this treatment.

In larger-scale fractionations 225 ml of dialysed rabbit serum was dialysed (with intermittent stirring) against a solution of ammonium sulfate of a concentration such that at equilibrium a 42.5% saturated solution resulted. This slow (7 days) precipitation produced relatively large aggregates which could be separated conveniently by filtration. The suspension was filtered and the precipitate was washed with 25 ml of cold 42.5% ammonium sulfate solution. The filtrate (containing the active material) was dialysed for 7 days, while changing the water twice daily, to remove excess salts. The small amount of precipitate was removed by filtration and the filtrate was dialysed for 7 days against a solution of ammonium sulfate of a concentration such that at equilibrium a 71% saturated solution resulted. The suspension was filtered and the precipitate (containing the active material) was washed with 50 ml of cold 71% saturated solution of ammonium sulfate. The precipitate was dissolved in



Ascending Descending

FIG. 1. Electrophoretic patterns of ascending and descending boundaries of rabbit serum albumin. The pH was 8.41 using barbitol buffer and the ionic strength .1. The determination was made over about 6 hr at 200 volts and 4.5 milliamperes. The authors are indebted to Dr. Sidney Roberts, U.C.L.A. Medical School, for these determinations.

TABLE I. Response of *Leptospira canicola* to Rabbit Serum Fractions.*

% ammonium sulfate saturation	Increase in optical density	
	Filtrate	Precipitate
14.2	31.6	‡
25.8	24.4	.0
42.5	23	.0
56.9	7.8	13.6
71 †	.0	17.6
Control (dialysed serum)	35	30
" (whole serum)	22.5	36.3

* 10% by volume of test material prepared in preliminary experiments was added to the chemically-defined basal medium. Filtrates and precipitates were assayed at different times.

† At higher concentrations the precipitate in the resulting viscous suspensions could not be separated by filtration or centrifugation.

‡ No precipitate formed.

‡ The authors are indebted to Mr. C. D. Boone (Puente, Calif.) for rabbit blood, Dr. E. J. Cohn for the crystalline human plasma albumin, the Lederle Laboratories, Pearl River, N. Y., for the crystalline sheep and crystalline bovine albumin, No. 2, and the Hyland Laboratories (Los Angeles, Calif.) for filtering and bottling the rabbit serum. For this purpose 100 ml aliquots of serum were placed in cellulose sausage casings (The Visking Corp. size 36/32) and the latter were suspended in 5-gal. bottles of distilled water. The bottles were allowed to stand in the refrigerator for 10 days during which the water was changed twice daily. The resulting dialysed serum was filter-sterilized and stored in sterile bottles in the refrigerator. The dialysed serum was added to media in amounts equivalent to a concentration of 10% whole serum.

§Strain No. 18 (Hond Utrecht IV) and *Leptospira icterohemorrhagiae,* Strain No. 20 (Wiznberg) were obtained through the courtesy of Dr. K. F. Meyer, University of California, San Francisco.

TABLE II. Response of *Leptospira canicola* to Rabbit Serum Albumin.

Albumin		Albumin	
Preparation No. 1,* ml/tube	Activity†	Preparation No. 2,‡ ml/tube	Activity†
.0	1	.0	0
.5	3	.2	8
1	6	.4	16
1.5	15	.6	33
1.7	19	.8	36
2	24	1	40
2.5	37	1.5	39
2.7	37		
3	38		
Control (dialysed serum)	34	Control (dialysed serum)	35

* Larger-scale preparation containing 2.75 mg N/ml.

† Optical density, each value average of 5 determinations.

‡ 6.9 mg N/ml.

water and reprecipitated twice in the same manner. Other preparations from as much as 2 liters of dialysed serum were made by analogous methods.

Results and discussion. The most active fraction of serum albumin was demonstrated to be electrophoretically homogeneous as shown by the patterns of the ascending and descending boundaries (Fig. 1).

As shown in Table I the growth of *Leptospira canicola* on the described chemically-defined basal medium with added filtrate decreased and with added precipitate increased with increasing concentrations of ammonium sulfate employed to fractionate proteins in the dialysed rabbit serum. According to the data in Table II growth of the spirochete increased with increasing concentration of the albumin fraction precipitated at 71% ammonium sulfate saturation. As indicated in Table III full growth of the organism was not maintained in serial subcultures on the chemically-defined medium lacking the amino acids (solutions A and G) but supplemented with the albumin. Since it was determined that 2.5 ml of the albumin fraction (Preparation 1) containing 6.9 mg of nitrogen was as effective in promoting growth of the spirochete as 0.8 ml of the dialysed rabbit serum (equivalent to 0.6 ml of the whole serum) containing 4.8 mg of albumin nitrogen, there was an approximate correlation between the activity of the albumin in the dialysed serum and its most active fraction isolated by ammonium sulfate precipitation. Although the active fraction may have been partially denatured the lack of exact equivalence in activity of these albumins may be explained, at least in part, if the estimate of nitrogen in the albumin of the dialysed serum were incorrect. In making this estimate it was assumed that the serum contained 5% albumin and the latter 16% nitrogen.

The activities toward *Leptospira canicola* of serum albumins obtained from bovine, horse, human and sheep bloods are tabulated

TABLE III. Response of *Leptospira canicola* Rabbit Serum Albumin in Different Media.

Group		Initial culture	Increase in optical density* Subculture 1	2	3	4	5	6	7
A Albumin†	C-D M	31	33	26	27				
Control‡	C-D M	33	33	38	34				
B Albumin§	C-D M	32	28	25	10	26	29	30	27
	D-W	45	22	18	0				
	(C-D M)-(AA)	49	40	23	18	23	23	14	3
Control‡	S-B	34	34	29	33	35	31	31	30
C Albumin§	C-D M	19	21	35	39	30	32	39	
	S-B	33	41	46	47	44	41	47	
Control‖	S-B	22	31	43	26	36	36		

* Each value is the average of 2 determinations in Group A, 4 in Group B, and 6 in Group C. The groups were not run simultaneously.

† 2.5 ml (2.75 mg N/ml) of preparation No. 1 used per tube.

‡ Dialysed rabbit serum.

§ .8 ml (6.9 mg N/ml) of preparation No. 2 used per tube.

‖ Whole rabbit serum (different lots).

C-D M, chemically-defined medium; D-W, distilled water; S-B, stock base (same as transfer medium).

TABLE IV. Response of *Leptospira canicola* to Different Serum Albumins.

Albumin	Activity**
1. Crystalline bovine [illegible]	[illegible]
2. [illegible]	[illegible]
3. [illegible] rabbit serum	[illegible]
4. Crystalline [illegible]	[illegible]
5. [illegible]	[illegible]
6. Salt-precipitated [illegible]	[illegible]
7. [illegible]	[illegible]
8. [illegible]	[illegible]
9. [illegible] ¶	[illegible]
10. [illegible] rabbit serum	[illegible]

* [illegible] obtained from [illegible] Laboratories, Chicago, Ill.

† [illegible] fractionated product obtained through the courtesy of [illegible].

‡ [illegible] obtained through the courtesy of [illegible] Laboratories, [illegible].

§ Prepared by the authors from whole blood obtained from the Los Angeles Wassermann Supply Co., Los Angeles.

|| Prepared by the authors from whole blood obtained from the Victory Slaughtering Co., Los Angeles.

¶ 1 or [illegible] mg [illegible] used per tube.

** Increase in optical density [illegible] average of [illegible] determinations after [illegible] incubation at 30°. The chemically-defined basal medium was used with [illegible] and the [illegible] medium supplemented with peptone and [illegible] with [illegible].

in Table IV. Generally, the crystalline and the alcohol-precipitated albumins lacked, or had low, activity while the salt-precipitated albumin fractions (except bovine) exhibited activity nearly comparable to that of the control (dialyzed rabbit serum). Savino and Renella(2) reported that hematin was a growth factor for *L. icterohemorrhagiae* but it seems unlikely that the growth effects observed in the present experiments are accounted for by heme compounds. It may be noted that traces of heme compounds were present in the inactive, as well as the active, fractions of the rabbit and horse sera.

In further experiments it was found that salt-precipitated horse or sheep albumin could substitute satisfactorily for dialyzed rabbit serum or salt-precipitated rabbit albumin in supplementing a basal medium containing only peptone and salts. Subculturing of *L. canicola* was not attempted although it was determined that *L. icterohemorrhagiae* could be propagated satisfactorily by transferring it weekly over a 3-month period in the peptone-salt-rabbit serum albumin medium. It is of interest that Chang(3) was unable to maintain *L. icterohemorrhagiae* in a medium in which horse serum was replaced by crystalline bovine serum albumin.

Summary. *Leptospira canicola* has been cultivated satisfactorily on a chemically-defined medium supplemented with electrophoretically-homogeneous rabbit albumin separated from serum by ammonium sulfate fractionation. The results of experiments with crystalline bovine and human serum albumins and with salt-precipitated albumin fractions from bovine, horse and sheep sera have been presented.

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Rate of Potassium Exchange in the Rat Erythrocyte.* (19216)

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Previous studies have shown that the human erythrocyte utilizes nearly a millimole of glucose for every millimole of potassium that enters the cell(1). The following experiments were undertaken to determine if a similar relationship existed in the metabolism of the rat erythrocyte.

Mature white rats of the Wistar strain were

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† National Research Council Fellow in the Medical Sciences.

killed by a blow on the head and the abdomen and chest were immediately opened. Blood from the heart or vena cava was drawn into a syringe containing heparin. Using the methods previously described(1), the blood was mixed with an isotonic medium containing inorganic salts and glucose. The final percentage of cells of the diluted whole blood was between 10 and 20. $K^{42}Cl$ was incorporated into the diluting medium as a tracer. The diluted whole blood was incubated at 35°C to 36°C in rocker-dilution vessels(2) and equilibrated with 5% carbon dioxide and 95% oxygen. Four or 5 samples were withdrawn periodically during the course of the 10 to 12 hour incubation period for determinations of the percentage of cells (Hct), the diluted whole blood potassium and radioactivity, the diluted plasma potassium ($[K^+]p$), and the diluted plasma radioactivity ($K^{42}p$). Glucose determinations on the diluted whole blood were also done at the beginning and end of the experiment.

As some leakage of the intracellular potassium occurred during the course of the experiments, the rate of entrance of potassium into the rat erythrocytes from the suspending medium was determined by plotting the log of the radioactivity of the diluted plasma compartment, $K^{42}p$ x (1-Hct), against time. The initial slope of the first hourly period was used to calculate the per cent of the radioactivity of the diluted plasma compartment entering the cells per hour. The number of millimoles of potassium exchanging per hour was calculated from this percentage by multiplying it by the number of millimoles of potassium present in the plasma compartment, $[K^+]p$ x (1-Hct). As determined in 8 experiments, the mean value for the number of millimoles of potassium exchanging per hour per liter of cells was 5.24. The mean value for the percentage of the erythrocyte potassium which was exchanging every hour per liter of cells was 4.99. Glucose was utilized at the rate of 2.47 millimoles per liter of rat red cells per hour. A summary of the data from these experiments is shown in Table I.

TABLE I. Amount of Potassium Exchanged and Glucose Utilized by Rat Erythrocytes.

Exp. No.	% of cell K exchanging per hr	mM of cell K exchanging per hr per l. of cells	mM glucose utilized per hr per l. of cells
1	4.10	3.50	
2	5.97	6.35	—
3	4.70	5.06	2.17
4	4.53	5.18	1.89
5	5.26	5.47	2.67
6	5.78	5.94	2.89
7	4.81	5.23	2.72
8	4.77	5.21	2.50
Mean	4.99	5.24	2.47

The number of millimoles of potassium exchanging per liter of red cells per hour for the rat is about 3½ times greater than that found for the human(1). On a cell surface area basis, the rat cell exchanges nearly three times as much potassium per unit time as does the human erythrocyte. These comparisons are shown in Table II.

Summary. (1) 5.0% of the intracellular potassium of the rat erythrocyte exchanges every hour, or 5.2 millimoles of potassium per liter of cells per hour. (2) 2.5 millimoles of glucose are utilized per hour per liter of rat erythrocytes. (3) The rat erythrocyte exchanges about 3½ times as much potassium per unit time as does the human erythrocyte. (4) The human erythrocyte utilizes nearly a millimole of glucose for every millimole of potassium that enters the cell. The rat erythrocyte utilizes less than ½ a millimole of

TABLE II. Comparison of Potassium Exchange and Glucose Utilization of Human and Rat Erythrocytes.

	mM K exchanging per l. red cells per hr	mM K exchanging per μ^2 surface area per hr*	mM glucose utilized per l. red cells per hr	mM glucose utilized per μ^2 surface area per hr*
Human erythrocyte	1.52	1.14×10^{-15}	1.39	1.05×10^{-15}
Rat "	5.24	3.26×10^{-15}	2.47	1.52×10^{-15}

* Calculated on the basis of the surface area of a human erythrocyte being 119 square microns and of the rat erythrocyte being 86 square μ (3).

glucose for every millimole of potassium that enters the cell.

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The Hemolytic Action of Gramicidin and Tyrocidin. (19217)

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Tyrothricin, an antibiotic complex isolated by Dubos(1) from the soil organism *Bacillus brevis*, consists of at least 2 hemolytic substances in the following approximate proportions: tyrocidin, 85%, and gramicidin, 15%. The hemolytic activities of these 2 substances as well as that of tyrothricin have been reported by various research workers(2-6). Quantitative data on the rates of reaction of the pure substances as well as mixtures are lacking, however. This paper presents comparative data on the hemolytic activities of gramicidin and tyrocidin and various mixtures. The analyses were made under the conditions developed by the author(7) for quantitative determination of tyrothricin.

Materials and methods. Stock alcohol solutions of the 2 purified substances† were made to contain 20 μg per ml of gramicidin or tyrocidin. Working solutions and mixtures were prepared by dilution with 95% alcohol.

One-half ml aliquots of the diluted solutions were added to 10 ml of a 0.25% twice-washed rat erythrocyte suspension in 0.87% saline, and the course of hemolysis was determined with a Klett-Summerson photoelectric colorimeter. A 660-millimicron filter was employed. Scale readings are proportional to optical density.

Action of gramicidin and tyrocidin. The course of hemolysis is markedly different for these two agents (Fig. 1). The hemolytic effect of gramicidin is represented by a sigmoidal curve with a pronounced lag period. The length of this period is dependent upon the concentration of gramicidin and the temperature. Tyrocidin, on the other hand, causes very rapid hemolysis, which is inhibited after about 10 minutes. The extent of hemolysis is dependent upon the concentration of the tyrocidin. Experiments discussed below indicate that the tyrocidin combines with some component of the cell stromata and becomes inactivated.

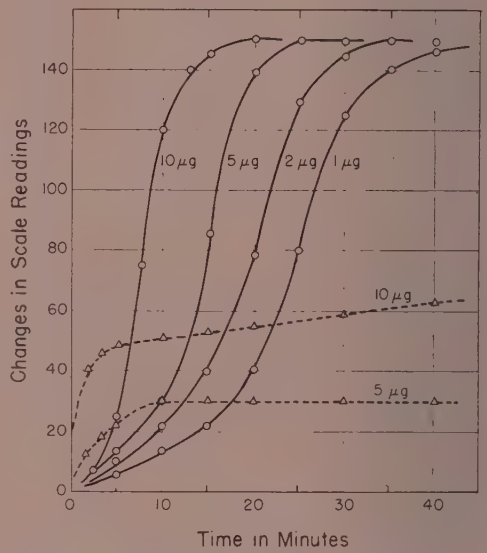

FIG. 1. Hemolysis curves for gramicidin and tyrocidin. Solid lines, gramicidin; broken lines, tyrocidin.

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† Several times recrystallized gramicidin and tyrocidin hydrochloride were prepared from crude tyrothricin according to methods of Hotchkiss and Dubos(4).

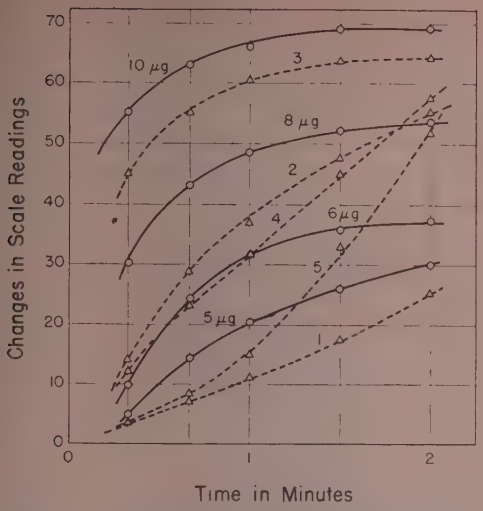

FIG. 2. Hemolysis curves for tyrothricin and for various mixtures of gramicidin and tyrocidin. Solid lines, natural tyrothricin.

Curve 1—	.0% grami',	100 % tyro',	8 μg total.
2—	4.8	95.2	8.4
3—	16	84	9.6
4—	23	77	7.8
5—	33.3	66.6	7.5

The hemolysis curves obtained with mixtures were found to be characteristic of the composition of the mixture in the early part of the reaction (dotted curves, Fig. 2). The solid curves represent the rate of hemolysis produced by various concentrations of a sample of tyrothricin which was isolated in this laboratory. The data for this sample agreed very well with those for other preparations made here, for samples obtained from commercial sources, and for a mixture of 16% gramicidin and 84% tyrocidin (see curve 3, Fig. 2). Hotchkiss and Dubos(4) have found, by chemical isolation, that tyrothricin is composed of approximately this same proportion of gramicidin and tyrocidin.

Although critical experiments have not been made, it should be possible to estimate the relative proportion of these 2 substances by determining the type of curve produced. It is easy to determine either of these compounds by the hemolytic procedure when the other is absent. Such measurements were made on chemically modified gramicidin in an attempt to correlate loss of hemolytic activity with loss of toxicity(11-13).

Tyrocidin inactivation. Tyrocidin appears to combine with some component of the red blood cell to inactivate its hemolytic activity. Several experiments were designed to investigate further the inactivating effect of the blood cells on tyrocidin and gramicidin.

Three or 4 times the amount of tyrocidin necessary to completely hemolyze was added to a sample of washed erythrocytes (1000 μg in 1 ml of cells) and allowed to stand for one hour. Alcohol was added to the completely hemolyzed solution to 90% concentration in order to extract the tyrocidin. Aliquots of this extract were mixed with a fresh aliquot of red cells, and no trace of hemolysis was observed, although an excess of tyrocidin had been added to the original erythrocyte suspension.

To test this possibility further, aliquots of a 90% alcoholic extract of carefully washed erythrocytes were added to an alcoholic solution of tyrocidin. No hemolytic action was obtained when the mixture was tested on fresh red blood cells. In order to determine whether this inactivating agent was present in the cell wall or in the water-soluble part of the cell, a sample of erythrocytes was laked with distilled water and the cell walls (ghost cells) were centrifuged off and washed twice with distilled water. An alcoholic extract of the cell walls was found to contain approximately all of the inactivating agent, while the water-soluble components of the cell had no inhibitory effect. A benzene extract of dried erythrocytes was found also to inhibit the hemolytic activity of tyrocidin.

Several investigators(8-10) have found that phospholipids will inhibit the bactericidal action of tyrothricin on bacterial cells, while Heilman and Herrell(2) have found that cholesterol does not neutralize the hemolytic effect of gramicidin. The inhibitory agent or agents obtained from blood cells is similar to phospholipids with regard to solubility characteristics. Similar experiments with gramicidin showed that it was inhibited, but to a much lesser extent, than tyrocidin. Inhibition of gramicidin and tyrocidin with regard to their bactericidal action was not studied.

Summary. (1) The courses of hemolysis for gramicidin, tyrocidin, and mixtures of the

two have been determined. Tyrocidin causes a rapid hemolysis, the extent being proportional to the concentration of the lysin. Gramicidin causes a slower initial rate of hemolysis but this initial lag is followed by rapid destruction of red blood cells at a rate at least equal to the initial rate due to tyrocidin. Mixtures of the two components gave hemolysis patterns characteristic of the proportions of gramicidin and tyrocidin. (2) An alcohol-soluble substance or substances present in the wall of the red blood cell of the rat, possibly phospholipid in nature, combines with tyrocidin, and to a lesser extent with gramicidin, causing them to lose their hemolytic activity.

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Serum Cholinesterase Activity of Dogs in Shock or after Hepatectomy.* (19218)

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Since the cholinesterase of the serum is probably derived in large part from the liver (1-3), its serum concentration may reflect hepatic function(4). Therefore, serum cholinesterase levels were measured as a part of a study of hepatic dysfunction in experimental hemorrhagic shock. For comparison these data were also obtained in hepatectomized dogs.

Methods. Hemorrhagic shock was produced in unanesthetized dogs, using the elevated reservoir bleeding technic previously described (5). Serum from arterial blood was obtained (a) before hemorrhage, (b) during hypotension before transfusion, (c) when hypotension recurred after blood replacement, and (d) just before death (Table I). Total hepatectomy was performed in 3 dogs by a one-stage method, preserving the integrity of the inferior vena cava(6). In one other dog the hepatic artery and portal vein were ligated and a portal-caval anastomosis was carried out without removal of the liver. In another, the hepatic artery alone was ligated. Serum from arterial blood was obtained before these procedures and at intervals thereafter. Serum cholinesterase and total esterase were determined colorimetrically, employing beta-carbonaphthoxy-choline iodide and beta-naphthyl acetate as the respective substrates

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† Research Fellow of the National Cancer Institute.

TABLE I. Course of Hemorrhagic Shock.

Dog No.	Bleeding vol (cc/kg)	Period of hypotension* before transfusion (hr)	Period from transfusion to relapse† (hr)	Subsequent survival (hr)
1	50	1.6	2	1.6
2	61	2.6	.8	.8
3	57	4.3	.8	2
4	71	3	3.2	2
5	44	3	.8	.5
6	52	6.2	1.5	2
7	52	1.8	4.3	.2
8	67	6.5	1	—
9	44	3.5	1	—
10	47	5		
11	60	8	—	"
12	40	7.5	—	"

* Arterial pressure 30 mm Hg.
† Post-transfusion relapse to arterial pressure 60 mm Hg.

TABLE II. Serum Cholinesterase Activity, Total Esterase Activity and Total Protein Concentration Before Bleeding (Control) and in Various Phases of Hemorrhagic Shock.

	Serum cholinesterase activity (units/100 cc)				Total esterase activity (units/100 cc)			
		Shock				Shock		
Dog No.	Control	1*	2†	3‡	Control	1*	2†	3‡
1	3	1.1	2.5	2.7	19.2	18.7	18.2	30
2	2.9	2.7	2	1.3	19.7	23.8	19.3	22.2
3	1.9	1.95	3	2.3	16.4	18	24.8	17.5
4	0	0	—	—	16.6	20.3		
5	0	0	—	—	19.3	8.4	—	-
6	0	0	0	0	13.2	21	25.5	26.3
7	0	0	0	0	4.9	15.5	25.8	25.5
8	0	0	0		23.5	12.8	26.1	
9	.6	1.9	1.6	—	21.5	26.4	36	
10	3.1	2.6	—	—	15.3	21.2		
11	2.4	2.9	—	—	16.1	19	—	—
12	2.8	1.1	—	—	14.9	15.1		
Mean	*2.4*	*2*	*2.3*	*2.1*	*16.1*	*25.4*	*24.5*	*25.2*
	Mean total serum protein conc. (g/100 cc)				6.5	6.7	6.6	6.7

* 1. At end of period of controlled hypotension; before transfusion.
† 2. At post-transfusion relapse into shock.
‡ 3. At death (see Table I).

(7,8). One unit of activity is defined(7) as that amount of enzyme which liberates 10 mg of beta-naphthol per hour from the substrate under standard conditions.‡ Total protein concentration was determined by the copper sulfate falling-drop method(9).

Results. (Tables II and III). No change in serum cholinesterase activity was noted during hemorrhagic shock or during a 24-hour period following hepatectomy or hepatic devascularization. In 6 of the 17 experiments, the control specimen showed no serum cholinesterase activity and none appeared thereafter.

A rise in *total* esterase activity of the serum occurred in most of the dogs during shock or after hepatectomy. Serum total protein levels did not change significantly.

Discussion. At least 2 enzymes are adapted to the hydrolysis of acetylcholine. One of these is acetylcholinesterase in nervous tissue and in the red cell. The other is serum cholinesterase, which in the dog is found in serum, pancreas, parotid, parasympathetic ganglia and myenteric plexus. The substrate we employed is specific for serum cholinesterase since it is not hydrolyzed by acetylcholinester-

‡ 1/40 cc of dog serum is usually required. Hemolysis increases total esterase activity of the serum, but does not alter the serum cholinesterase activity as determined by this method.

TABLE III. Serum Cholinesterase Activity, Total Esterase Activity and Total Protein Concentration Before Operation (Control) and at Intervals After Hepatectomy or Hepatic Devascularization.

		Serum cholinesterase activity (units/100 cc)				Total esterase activity (units/100 cc)			
			Postoperative				Postoperative		
Dog No.	Preparation	Control	4-6 hr	8-12 hr	16-24 hr	Control	4-6 hr	8-12 hr	16-24 hr
H1	Hepatectomy	2.2	2.1	2.3	2.4	21.6	21.8	24.3	27.5
H2	"	.9	—	—	.9	16.2	—	—	19.4
H3	"	1.7	2.1	—	4.1	24.8	18	—	36.8
H4	Hepatic artery divided. Eck-fistula	4.6	4.4	4.4	4.4	23.1	22.5	—	27.4
H5	Hepatic artery divided.	0	—	—	0	28.5	—	—	29.4
*Mean**		*2.4*	*2.7*	*3.3*	*2.6*	*21.4*	*20.8*	—	*27.4*
	Mean total serum protein conc. (g/100 cc)					*6.3*	*6.2*	*6.3*	*6.1*

* Does not include H5.

ase of nervous tissue or the red cell or by other aliesterases of serum(7).

The physiologic significance of serum cholinesterase is obscure. In the rat its activity can be reduced by feeding tri-ortho-cresyl phosphate to 20% of the normal level without noticeable physiologic effect(10). The average level in normal dogs was one-tenth the amount found by the same method in human serum(7), and in some apparently normal dogs none could be detected.§ The absence of a decline in serum cholinesterase activity during hemorrhagic shock or after hepatectomy suggests slow utilization of the enzyme. The blood level of this enzyme, therefore, is not a sensitive index of acute changes in hepatic function in the dog. The findings are in agreement with those of an earlier study of serum cholinesterase activity during hemorrhagic shock in the dog(11), and with the delayed fall in serum cholinesterase in rats following acute liver damage produced by carbon tetrachloride(3). Guinea pig serum cholinesterase activity is reported to decline following trauma(13). In these earlier studies acetylcholine was used as substrate, so that serum cholinesterase alone was not specifically measured.

Plasma fibrinogen concentration does not fall during hemorrhagic shock or after hepatectomy in the dog(14). On the other hand, the prothrombin activity of dog plasma declines in proportion to the degree of deterioration during shock(15).

The administration of cholinesterase to dogs in hemorrhagic shock has been found ineffective(11). A reported favorable effect of cholinesterase given to dogs subjected to intestinal trauma cannot be evaluated in terms of an effect upon shock because the test dogs were also under the influence of thyroid hormone and barbiturate anesthesia(11).

The total esterase activity of the serum toward beta-naphthyl acetate includes the activity of ali-esterases(7), cholinesterases(7), and lipases(16) arising from pancreas, liver, blood cells and other tissues. No explanation is apparent for the rise in total esterase activity.

Conclusions. (1) Serum cholinesterase activity of normal dogs is approximately one-tenth that of normal human serum as measured by a colorimetric method employing beta-carbonaphthoxy-choline iodide as substrate. (2) No change occurs in the serum cholinesterase activity of dogs during hemorrhagic shock, or during 20 hours of survival after hepatectomy.

§ Technics using acetylcholine as substrate do not yield such low values in dog serum(11,12).

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Further Studies on Formate Incorporation by Leukemic Blood Cells.* (19219)

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It has been observed that at one hour after injection of C^{14}-formate into mice with advanced Ak-4 leukemia, the greater portion of the leukemic prolymphocytes give positive autoradiograms on nuclear track emulsion while very few normal-appearing lymphocytes and practically none of the polymorphonuclears or erythrocytes are positive(1). Two preliminary explanations of these results were considered: (a) that the C^{14}-formate was incorporated during the anabolic phases of cell division and that the leukemic cells were dividing very rapidly as compared to normal cells, or (b) that the formed leukemic cells were metabolizing formate much more rapidly than were normal blood elements. The present study was designed to obtain information which might shed light on this question. In these experiments, leukemic blood has been exposed to C^{14}-formate away from sites of hematopoiesis for subsequent autoradiographic examination.

Experimental. Blood was aspirated from the heart of Akm mice with advanced transplanted Ak-4 leukemia (a rather acute lymphoid strain). This blood was heparinized and placed in a small cellophane bag which was then introduced through an incision into the abdominal cavity of leukemic mice (Ak-4). These mice (with leukemic blood-containing cellophane bags) were injected IP. with 100 microcuries of C^{14}-formate. After one hour, the mice were sacrificed and blood smear autoradiograms were prepared on cardiac and cellophane-bag blood after each had been washed twice with inactive sera. The details of the procedure employed in making the autoradiograms have been presented(1). A number of blood smears on NTB plates (10 μ emulsion thickness) were made on each sample. The plates were developed for 2 or 20 minutes after exposure periods in the dark from 1 to 10 weeks. All of the NTB plates were examined under the microscope and a hundred or more cells on each were classified as grossly positive or negative. The results of these experiments are summarized in Table I.

Discussion. The white blood counts on the leukemic host animals in Exp. No. 1 and 2 were 7,500 and 34,800, respectively, somewhat

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TABLE 1. Summary of Autoradiogram Results on Cardiac and Cellophane-Bag Blood.

Exp. No.	Blood	Lymphocytes, 6-9 μ		Large lymphocytes and prolymphocytes, >10 μ		Segmented cells	
		Fraction positive	% positive	Fraction positive	% positive	Fraction positive	% positive
1	Cardiac	16/428	3.7	98/192	51	0/198	0
	Cellophane bag	5/261	1.9	62/162	38	0/200	0
2	Cardiac	12/240	2.9	67/120	56	0/214	0
	Cellophane bag	3/120	2.5	62/134	46	0/255	0

In Exp. 1, the donor supplying the bag blood and the host animal were in the fifth leukemic day. Mice used in Exp. 2 were in the sixth leukemic day. Cells were counted in many randomly picked fields. All cells in each field were classified as positive or negative. Two observers counted each slide with rather good reproducibility.

lower than animals used in earlier experiments (1). The values of 51% and 56% positive large lymphocytes plus prolymphocytes (cells larger than 10 μ in diameter) in the "host" mice are lower than the 90% positive prolymphocytes previously reported on cardiac blood in leukemic mice.

The per cent positive large lymphocytes and prolymphocytes in the cellophane bag blood was only slightly less than observed in cardiac blood from the "host" mice. This would seem to suggest that the profound difference between formate utilization in normal lymphocytes and leukemic prolymphocytes previously reported (at one hour after injection of C^{14}-formate) is a result of the more rapid uptake of the radioactive compound by formed leukemic cells. It seems unlikely that blood cells immobilized in a cellophane bag in the abdominal cavity of a mouse are proliferating as rapidly as peripheral blood cell precursors; however, this possibility cannot be excluded by the present results.

Summary. Experiments have been carried out which demonstrate that leukemic cells immobilized in a cellophane bag implanted in the abdominal cavity of a host mouse incorporate C^{14}-formate much more rapidly than do normal lymphocytes.

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Life Span of the Red Blood Cell of the Rat Following Acute Hemorrhage.* (19220)

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Neuberger and Niven(1) showed with N^{15} labeled glycine in rabbits made anemic by bleeding that the life span of the red blood cell was considerably shortened. These findings are of sufficient interest to investigate for confirmation in the rat.

Methods. A series of 10 adult female rats of a highly inbred Curtis-Dunning strain was given 20 microcuries (1.64 mg) of glycine-2-C^{14} intraperitoneally 6 hours after an acute hemorrhage of 5 cc by cardiac puncture. For control, 10 female rats of the same species were given the same amount of glycine-2-C^{14}. The rats were handled, and the blood samples obtained and analyzed in the manner described previously(2).

Results. Fig. 1 shows the average specific activity of the hemoglobin of the rats as a function of time after administration of the C^{14} labeled glycine. This shows that in the

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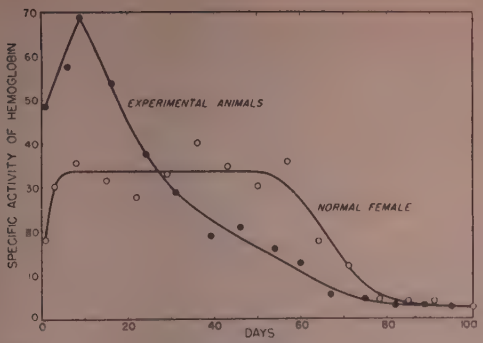

FIG. 1. Average specific activity of hemoglobin of anemic rats and normal rats following administration of glycine-2-C^{14}.

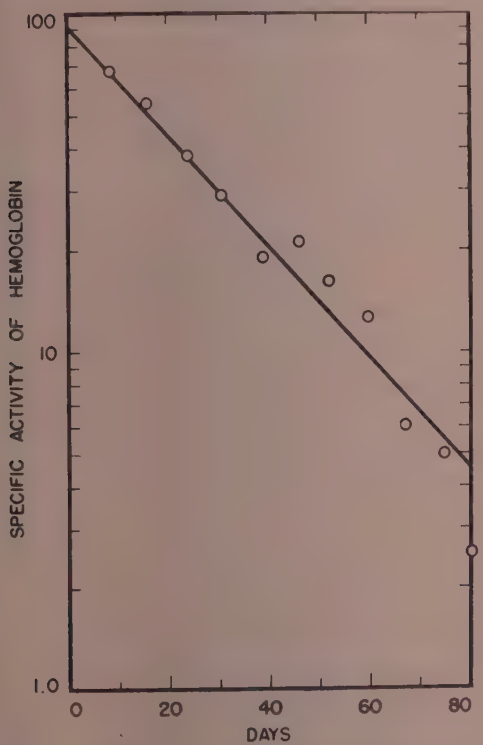

FIG. 2. Rate of fall in specific activity of hemoglobin of experimental animals from Day 10 to Day 70.

anemic rat the C^{14} level reaches a maximum and then, in contrast to the normals, falls rapidly.

Fig. 2 shows that in the anemic animals the rate of fall from day 10 to day 70 can be fitted to a single exponential component having a half-time of 19 days. This would indicate that these cells had a mean life span of 27 days. This value cannot be estimated by the method of Shemin and Rittenberg(3) but merely follows from the fact that the mean life time is 1.44 times the half-time when any process is a simple exponential decay process (4), such as this seems to be. Fig. 1 also shows that there is an approximately 2-fold increase in the amount of C^{14} incorporated in the red blood cells of the anemic animals. The control series of 10 female rats showed almost the same mean red cell life span as the male rats of a genetically similar strain, or 64 days as compared to 68 days(2).

Discussion. Following hemorrhage the red blood count and hemoglobin have returned to normal in 8-15 days (5-9); thus little, if any, of the decrease in the specific activity of the hemoglobin after day 10 can be attributed to dilution resulting from an increased total red cell volume.

The finding of a mean life span of the red blood cell of 27 days in those red cells produced in response to acute hemorrhage in the rat extends the finding of Neuberger and Niven(1) from the rabbit to the rat. These data in the rat and rabbit suggest that the red blood cells produced in response to acute hemorrhage are functionally poor from the standpoint of life span, although adequate in numbers to replace rapidly the red cells lost. For the considerable period of time that these faulty cells are gradually being replaced by red cells having a more normal life span, the bone marrow will be hyperactive. In light of this evidence long-term studies of the transfusion of red cells from one animal to another in which the donor animal has previously undergone repeated bleeding are apt to be misleading.

Conclusion. The red blood cells formed as a response to acute hemorrhage in the rat have a considerably shortened life span.

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Effect of Ultracentrifugal Fractions of Small Intestinal Tissue upon Transplanted Lymphosarcoma.*† (19221)

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(Introduced by J. F. Mead.)
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The literature contains numerous reports of attempts to isolate from various normal tissues substances which are inhibitory to the growth of malignant tumors. The majority of these reports indicate highly variable results. Since it is generally known that malignant tumors rarely originate in the small intestine, the present work has been concerned primarily with efforts to derive inhibitory material from this organ. Previous work with this tissue has involved chemical extraction of material from the whole organ(1). In the present studies, ultracentrifugal fractionation has been employed. Similar methods have been used successfully in the separation of hemolytic and antihemolytic substances from liver(2).

Results of preliminary investigations based on centrifugal fractionation are reported in this communication.

Materials and methods. Small intestinal tissue of normal mice or rats was frozen on dry ice, then thawed and ground with sand. A saline suspension of this homogenate was fractionated in the following manner. A fraction corresponding to the mitochondrial-nuclear fraction of normal tissue(3) was separated by centrifugation for 10 to 20 minutes at 4000 x g (Fraction 4). The supernate from this was further centrifuged for 45 to 60 minutes at 90000 x g to yield a microsomal fraction (Fraction 90). The fractions were prepared for use by resuspending in saline. Fractions 4 and 90 and the supernatant from the latter were tested for inhibitory action on lymphosarcoma (Gardner). Lymphosarcoma cells in saline suspension were incubated *in vitro* at 5°C for 10 to 20 minutes with an equal volume of the tissue fraction suspensions described above. Two-tenths ml of these tissue fraction-tumor cell incubates was inoculated subcutaneously in CBA mice (Strong). Controls received saline-tumor cell inoculations.

Results. Results are summarized in Tables I and II. The technic employed yielded 100% takes of the tumor in 124 control mice. Tumors developed to palpable size in 4 to 7

TABLE I. Results of Pre-Inoculation Treatment of Lymphosarcoma Cell Suspensions with Mouse Tissue Fractions. Subcutaneous Injection of Tumor Cells and Tissue Fractions in CBA Mice.

Tissue fraction		No. cases	Survivals	Deaths
Intestine:	Fraction 90	49	47	2
	" 4	13	9	4
	Supernatant*	51	16	35
Liver:	Fraction 90	8	0	8
	" 4	8	0	8
	Supernatant	8	0	8
Spleen:	Fraction 90	6	0	6
	Supernatant	6	0	6
Muscle:	Fraction 90	6	0	6
	Supernatant	6	0	6
Controls:	Tumor + saline	65	0	65

* Supernatant fraction in all cases was the fluid portion remaining after precipitation of Fraction 90.

* This paper is based on work performed under Contract No. AT-04-1-GEN-12 between the Atomic Energy Commission and the University of California at Los Angeles.

† Portions of this study were carried out in the Department of Radiology, School of Medicine, and were partially supported by a grant from the University of California cancer research funds.

TABLE II. Results of Pre-Inoculation Treatment of Lymphosarcoma Cell Suspensions with Rat and Rabbit Small Intestinal Fractions. Subcutaneous injection of tumor cells and tissue fractions in CBA mice.

Tissue fraction		No. cases	Survivals	Deaths
Rat intestine:	Fraction 90	20	19	1
	Supernatant	20	0	20
	Controls	11	0	11
Rabbit "	Fraction 90	26	0	26
	" 4	6	0	6
	Supernatant	16	0	16
	Controls	48	0	48

days and death ensued in 17 to 27 days after inoculation. Death in all instances was due to uncontrolled lymphosarcoma. The treated mice have survived as long as 180 days after inoculation in good condition and with no evidence of tumors.

From the data it is evident that the maximum inhibition occurs in animals inoculated with tumor cell suspensions incubated with Fraction 90. Instances of survival in animals given tumor cell suspensions exposed to Fraction 4 or to the supernatant from Fraction 90 occurred in early experiments. Data from later experiments suggest that these survivals were due to overlapping during the fractionation procedure. Improved centrifugation technics in subsequent experiments have eliminated such results.

Corresponding fractions prepared in identical fashion to the above from other normal tissues (spleen, muscle, liver) showed no inhibitory properties. Fractions prepared from rabbit small intestine were ineffective, though this may be due to technical insufficiencies rather than to the absence of inhibitor.

In order to establish further that the inhibitory activity is due to specific substances in Fraction 90 rather than to bacterial contamination, proteolytic enzymes, mechanical or irritating effects or pH differences, appropriate experiments have been conducted to rule out these factors. Fraction 90 appears to be non-toxic since large intraperitoneal doses yielded no deleterious effects.

A limited number of experiments using Sarcoma 180 as the stock tumor indicated a response similar to that found with Gardner lymphosarcoma.

The data reported suggest that a non-toxic substance inhibitory to the development of lymphosarcoma is present in mouse and rat small intestine and that this material is recoverable by ultracentrifugal fractionation.

Further experiments are in progress to determine the nature of the inhibitory substance and to prepare it in a manner that will permit treatment of animals bearing established tumors.

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Stabilizing Action of Glycerine on Hemagglutination of Egg-Adapted Mumps, Newcastle Disease and Influenza Viruses. (19222)

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It has long been recognized that glycerine either destroys or inhibits certain bacterial contaminants and it has been extensively used as a bacteriostatic and preservative agent in virus infected tissues since its introduction by Copeman(1) for the purification of calf lymph vaccine. Levaditi and Harvier(2) reported that herpes, vaccinia, rabies, and poliomyelitis viruses remained virulent for weeks and even months *in vitro* when placed in pure glycerine or glycerine diluted with an equal volume of normal saline and stored at 4°C. Their findings were confirmed by Doerr and others(3,4). According to Rivers(5) the preservative ac-

tion of glycerine probably depends upon the inhibition it exerts on autolysis of virus infected tissues. Various authors have also reported the preservation of viability of some bacterial agents in pure glycerine for several years. McKinley and Verder(6) cite the preservation of green streptococci in glycerine for as long as four years. The same authors report a personal communication by Francis stating that he had maintained viability and virulence of *B. tularense* in 100% glycerine for more than 6 years and *B. pestis* for over 7 years, when the glycerinated preparations were kept in the cold. Glycerine has also been employed for the preservation of anti-sheep-cell hemolysin for serological work. It has been used as a preservative in the preparation of certain bacterial antigens, as for example the *B. abortus* stained antigen used in the ring test for Brucellosis(7). Takaki and others(8) used a glycerine extracted saline suspension of rabies infected brain as antigen in the complement-fixation test. So far as we are aware, however, there are no other references in the literature to the use of glycerine for the preservation and stabilization of viral sero-diagnostic antigens. Formalin and phenol, on the other hand, have been widely used against bacterial contaminants in the preservation of sero-diagnostic antigens; while these compounds are effective in checking bacterial growth they may adversely affect both sero-diagnostic and immunizing antigenic properties and titers eventually decrease despite such precautions as cold storage. The use of the hemagglutination-inhibition test as a diagnostic tool in testing for certain red cell agglutinating viruses is somewhat limited by the fact that the hemagglutinating property of the viral antigen may decrease quite rapidly upon storage at 4°C. In such cases a relatively fresh supply of infected material must always be kept on hand.

The purpose of this paper is to report some experimental observations on the stabilizing action of glycerine on the hemagglutinating activity of chick embryo propagated mumps, Newcastle disease and influenza viruses.

Materials and methods. Virus strains. All strains of virus included in this study were adapted to grow in the allantoic cavity of embryonated hen's eggs. Two strains of mumps virus were used: Strain H*(9) in its 25th egg passage and strain E*(10) in its 42nd passage in eggs. The Newcastle disease virus employed in this series of experiments was from a supply pool representing the second egg passage of the Isele strain isolated in these laboratories from a field case of the disease. The 13th egg passage of the PR^8 strain of influenza virus was also included in this study.

Methods of concentration. When concentrated virus suspensions were used the concentration was carried out by either one of two methods: Sharples centrifugation, as already described by others(11-13), or alcohol precipitation as described by Cox *et al.*(14) for the concentration and purification of influenza virus. *Titration method.* All virus samples were titrated by the Salk(15) hemagglutination procedure, using a 0.25% chicken red cell suspension. *Glycerine and saline.* The glycerine used throughout this study was a chemically pure, neutral, reagent glycerine having a specific gravity of 1.24. All glycerine mixtures were made gravimetrically rather than volumetrically in order to avoid errors from loss of glycerine on the pipette wall. Control mixtures were made in buffered saline(16) at pH 7.0. Formalin in 0.1% concentration was added to all saline and glycerine mixtures.

Experimental. Exp. I was set up in order to determine the effect of a 50% glycerine concentration on the hemagglutinating factor of egg-propagated mumps virus, when virus-glycerine mixtures were held at 4°C, 37°C and room temperature (22° to 24°C). Three preparations of each of the 2 mumps strains (E and H) were used: unconcentrated infected allantoic fluids, Sharples centrifuged concentrates and alcohol precipitates. Each of these virus suspensions was treated in the following manner: 60 ml of infected material were divided into 2 equal portions. To the first par, 30 ml of buffered saline were added; this mixture served as a control. The second part was mixed with 30 ml of undiluted glycer-

* The authors are indebted to Dr. Karl Habel for the H strain and to Drs. Levens and Enders for the E strain.

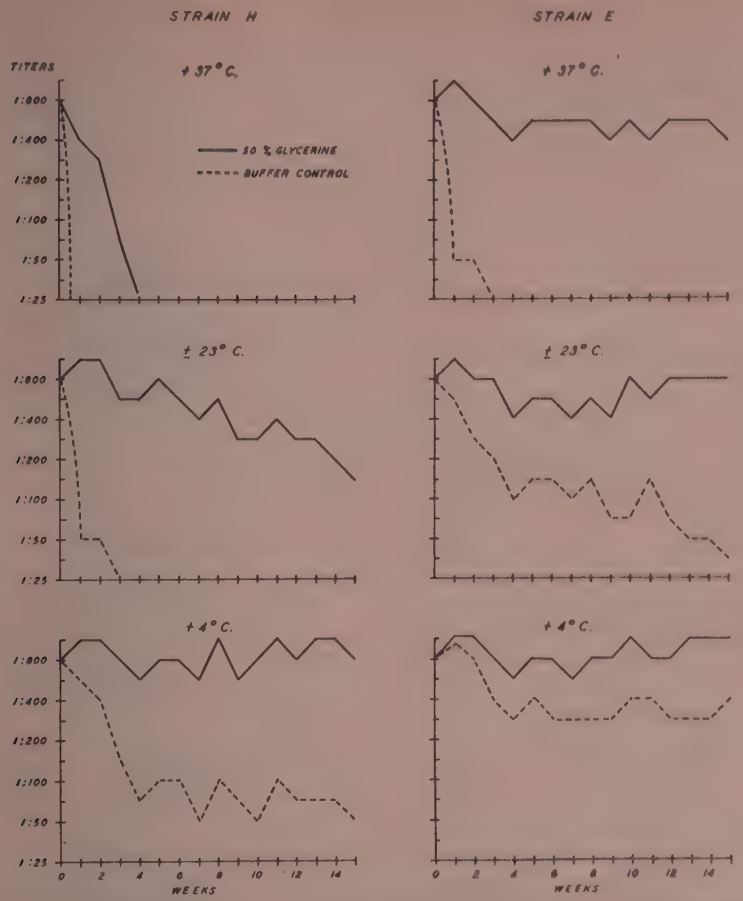

FIG. 1. Action of 50% glycerine on the hemagglutinin of mumps virus at three different temperatures.

ine, thus bringing it to a 50% concentration. Enough formalin was added to both buffered saline and glycerine mixtures to give a final concentration of 0.1%. Formalin was added mainly to protect the buffered saline suspension against bacterial action and was also used in the glycerine mixture in order to equalize the 2 preparations. Each of these 2 preparations was in turn divided into three portions of 20 ml each and these were placed at 4°C, 37°C and room temperature respectively. Titration of hemagglutinin was done at weekly intervals for 16 weeks, all samples being replaced at their respective temperatures as soon as enough material had been taken out for the day's test.

No differences in the titer behavior were observed among the three types of material used. To avoid repetition only the results obtained with the Sharples centrifuged concentrates of both mumps strains have been summarized in Fig. 1.

It can be seen that the hemagglutinating principle of mumps strain H is very labile in buffered saline and is destroyed overnight at 37°C. At room temperature its titer decreases quite rapidly during the first week and becomes completely negative by the third week. It is somewhat more stable at 4°C, but reaches an appreciably lower titer by the fourth week. With the addition of glycerine, however, the sample maintained at 37°C retained its he-

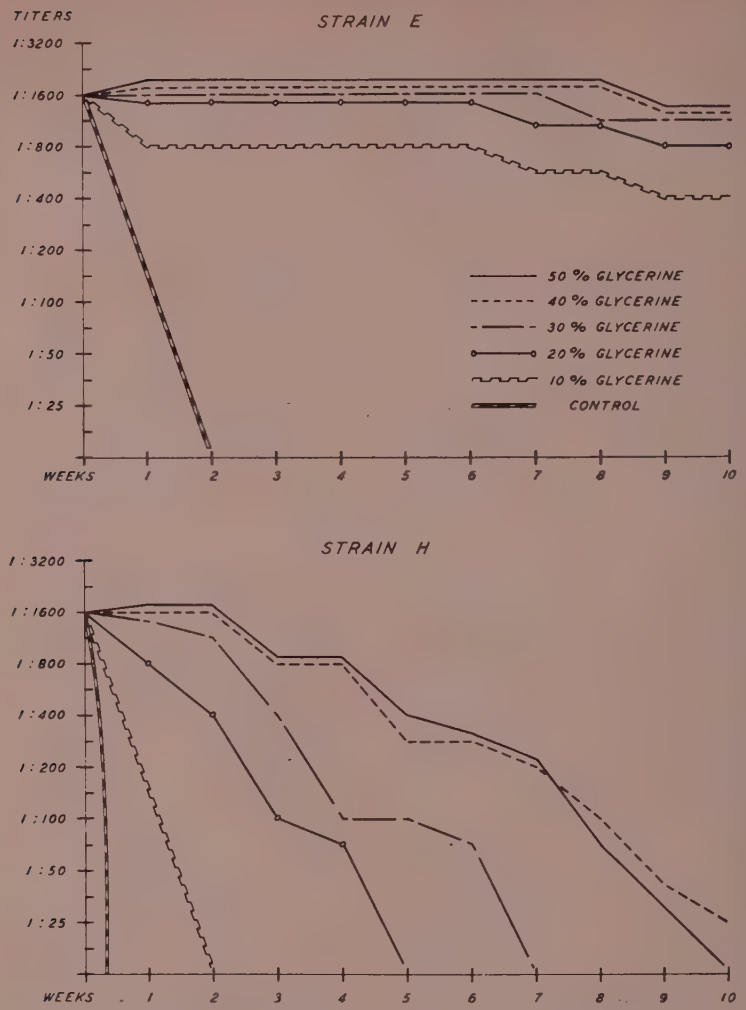

FIG. 2. Action of increasing concentrations of glycerine on the hemagglutinin of mumps virus at 37°C.

magglutinating activity for 4 weeks. The room temperature sample decreased in titer at a much slower rate and was still appreciably active after 16 weeks, while the 4°C specimen maintained its titer for the total observation period of 16 weeks.

The hemagglutinating capacity of mumps strain E is much more stable than that of strain H, even in buffered saline and lost its activity completely only after 3 weeks at 37°C. Its titer decreased at a slower rate at room temperature but reached a very low level after 16 weeks. At 4°C, it suffered only a 2-fold decrease after four weeks and then maintained the same level to the end of the experiment. The addition of glycerine seemed to preserve the titer of strain E just as well at all 3 temperatures.

Exp. II was devised to determine the lowest concentration of glycerine capable of stabilizing hemagglutinin titers. Sharples centrifuged concentrates were prepared from both E and H strains of mumps virus. Each batch was divided into 6 parts and glycerine mixtures of increasing concentrations from 10 to 50% were made; the sixth part was made up in

buffered saline to serve as a control. Formalin was added at a concentration of 0.1% to all 6 preparations of each strain. All mixtures were placed at 37°C and titrations were made at weekly intervals for 10 weeks. Results for both strains E and H are summarized in Fig. 2.

The lability of strain H again can be seen, since glycerine concentrations of 10, 20 and 30% delayed deterioration only partially. Even with 40 and 50% glycerine concentrations the titers became completely negative after nine to ten weeks. In contrast, 10% glycerine was enough to stabilize strain E to an appreciable extent and as little as 20 per cent was sufficient to maintain it practically at the same level for 10 weeks at 37°C.

Similar but less detailed observations carried out on suspensions of Newcastle disease and influenza (PR8) viruses, prepared in the same manner as the mumps virus suspensions, indicate that the hemagglutinating capacity of these viruses is also stabilized to a considerable degree by the addition of 50% glycerine. Buffered saline suspensions of Newcastle disease virus suffered a 128-fold loss of titer in less than 2 weeks at 37°C, whereas under the same temperature conditions those containing 50% glycerine showed not more than a 2-fold decrease in titer during a 5-week interval. Deterioration without glycerine was less rapid at room temperature than at 37°C, but a 32-fold drop in titer was observed in 4 weeks while at the same temperature the glycerinated sample had fallen by only a single 2-fold dilution at the end of 8 weeks. It was also observed that a suspension of PR8 influenza virus containing 5% glycerine suffered a 32-fold loss in titer at 37°C during an interval of approximately 8 weeks, while a comparable preparation containing 50% glycerine showed only a 4-fold drop in titer under the same temperature conditions.

It may be of interest to note here that a one liter batch of mumps antigen, prepared by the Sharples centrifugation method in October, 1949, and containing 50% glycerine, has maintained its original titer up to the present date, October 1951. During these 2 years, it has been constantly stored at 4°C. Similarly, a batch of Newcastle disease diagnostic antigen prepared with 50% glycerine had a titer of 1:3200 in November, 1949, and its titer in September, 1951, was 1:2240. This lot has been held at 4°C but has been in continuous use for routine tests during most of the 2-year interval.

Conclusions. (1) Experimental observations based on the behavior of two strains of mumps virus having hemagglutinins of markedly different stability show that the addition of glycerin has a marked stabilizing action on the hemagglutinating antigen. Without glycerine, at 37°C the H strain of mumps virus in buffered saline containing 0.1% formalin, loses its hemagglutinating activity overnight and the E strain in 3 to 4 weeks. In contrast, the H strain in 50 per cent glycerine suspension, also containing 0.1 formalin, maintained useable titers for 2 to 3 weeks at 37°C, and under the same conditions the E strain showed little or no loss of activity at the end of 16 weeks. (2) Glycerine concentrations as low as 10% markedly enhanced the stability of the E strain while the stability of hemagglutinating activity of the H strain was dependent to a much greater degree upon an increased concentration of glycerine. (3) The hemagglutinating activity of antigens prepared from suspensions of Newcastle disease virus and influenza virus (PR8) was similarly stabilized by the addition of 50% glycerine.

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Separation, Concentration, and Transfusion of Platelets.* (19223)

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Evaluation of the therapeutic usefulness of blood platelets has been hindered by the lack of a method which permits the efficient separation and concentration of these elements physiologically intact in quantities suitable for transfusion. The method described below has met these requirements reasonably well, and may serve as a useful point of departure for others interested in this field. It has been kept sufficiently simple that no unusual technical skill or equipment are required.

Materials and methods. The method is based on the use of di-sodium ethylene diamine tetra-acetate (Sequesterene Na_2†) as anticoagulant, and the use of differential centrifugation for the separation and concentration of the platelets. One g of Sequesterene and 0.7 g of sodium chloride were dissolved in 100 cc of distilled water. Nine parts of blood were added to one part of the Sequesterene solution. Venous blood was collected from humans, and arterial or venous blood from dogs, by gravity flow through a coated 15 gauge nylon-hub electro-polished needle‡ and plastic tubing‡ into 200 ml siliconed centrifuge bottles, each containing 20 cc of the Sequesterene solution. The guinea pig blood was aspirated from the heart into a syringe containing the Sequesterene solution and transferred to the centrifuge bottles. The bottles were carefully balanced and centrifuged at 30 x g for 50 minutes at 5°C. The supernatant platelet rich plasma was then siphoned into another 200 ml siliconed glass centrifuge bottle, using siliconed glass and plastic tubing as the siphon. This plasma was centrifuged at 300 x g for 50 minutes at 5°C, and the supernatant siphoned off leaving the platelets as a loosely packed mass in the bottom of the tube. The platelets were then resuspended in an aliquot of the supernatant plasma. Resuspension was readily accomplished by aspirating and expelling the platelet-plasma suspension 3 or 4 times in a siliconed sterilized cotton plugged pipette with a tip bore of 1 mm or more. All platelet counts were done by the method of Brecher and Cronkite(1). To test the viability of the platelets, animals were usually given the equivalent of their normal total platelet content by transfusion, the volume of suspension varying from 1-2 ml in guinea pigs and 10-25 ml in dogs. Sixty-three transfusions of pooled platelets were given to guinea pigs, and 19 were given to dogs; 4 of the guinea pigs were normal, 13 were irradiated, 3 of the dogs were normal, and 3 were irradiated. The transfusions were given within one hour of the preparation of the suspension and within 4-5 hours of drawing the blood. No platelet transfusions were at-

* The opinions and assertions are those of the authors and are not to be construed as reflecting the policy or opinions of the Naval Service.

† Supplied by courtesy of the Alrose Chemical Co., Providence, R. I.

‡ Product of the Fenwal Co., Ashland, Mass.

TABLE I. Results of Platelet Transfusions on Platelet Levels of Normal and Irradiated Dogs.

Dog No.	Post irradiation day	Platelets count, 1000/mm³ Before transfusion	Platelets count, 1000/mm³ 2 hr after	Total platelets inj., × 10^{10}	Vol susp., ml
257	Normal	365	395	1.6	25
334	"	300	365	2	25
335	"	300	365	6	25
335	11	8	20	18	30
335	12	40	140	13	20
335	13	58	125	11	30
335	14	115	190	21	21
337	15	60	180	12	20
338	5	295	285	6	20
338	6	190	275	10	20
338	7	165	225	7	20.5
338	8	175	205	17	22.5
338	9	125	200	15	22.5
338	10	130	120	3*	21
338	11	53	130	8	20
338	12	30	148	28	23
338	13	70	185	46	24.5
338	14	90	235	33	25

* Clumping observed in suspension.

tempted in humans.

Results. Centrifugation of whole blood as described above resulted in packing the red cells until they occupied a volume of approximately 60% of the whole blood. The white cells, along with some platelets, were above the red cell mass as a loosely packed buffy coat. The supernatant plasma contained platelets and an occasional small lymphocyte. The platelet count of this plasma was usually double the platelet count of whole blood in the case of human and guinea pig blood. With dog blood the platelet count was the same as that of the whole blood or only slightly higher. The use of a 1.5% Sequesterene solution instead of the usual 1% solution did not increase the platelet concentration in the dog plasma. Platelets not accounted for in the supernatant plasma could, however, be recovered by resuspending the buffy coat and the top 10% of the red cell mass in plasma, and repeating the first centrifugation. These platelets were not clumped, and could be readily concentrated and resuspended. Generally, not all of the plasma could be siphoned off without disturbing the buffy coat, hence a constant loss of supernatant plasma of about 10% was encountered. All of the platelets from the supernatant could be recovered in the second centrifugation if the tubes were well balanced. The recoveries of platelets from whole blood were: 50% for dog blood, 70% for human blood, and 85% for guinea pig blood. Examination with the phase microscope of the platelets in whole blood, supernatant plasma or in the final suspension, revealed no morphologic alteration during processing. In the final suspension there was no tendency to clump or to adhere to unsiliconed glass surfaces.

In vitro the blood of irradiated thrombopenic dogs has a prolonged clotting time of 30 minutes to several hours, forms a fragile non-retractile clot, and fails to utilize prothrombin. The addition of platelets prepared with the present method caused clotting in less than 2 minutes, utilization of prothrombin and clot retraction(2). No comparable studies were made with guinea pig blood. *In vivo* 20 to 70% of the transfused platelets were present in the peripheral blood of normal as well as irradiated dogs 2 hours after intravenous transfusion. Approximately 50% of the platelets were still present 24 hours later as judged from the platelet levels observed on the following day before the next transfusion was given (Table I). In the irradiated thrombopenic dog whose platelet level was maintained at approximately 100,000 per cu mm by platelet transfusion, the coagulation defect and the hemorrhagic tendency were prevented(2).

In guinea pigs with an average weight of

TABLE II. *Effects of Platelet Transfusions on Platelet Levels in Guinea Pigs.**

Platelet preparation lot and platelets per mm³ × 1000	A 4200		B 3500 (heated to 40°)			C 6300		D 7000			E 3000	F 7000	
Animal No.	1	2	3	4	5	6	7	8	9	10	11	12	13
Platelet count before transfusion × 1000	170	210	260	200	170	20	40	80	10	220	160	270	150
Platelet count 30′ after transfusion × 1000	400	350	240	140	200	110	170	260	50	390	240	270	700

* Animal No. 1-9, irradiated, transfused with 1 cc suspension. No. 10-13, normal controls, transfused with 2 cc of suspension.

250 g the average blood volume was estimated to be 20 ml (8% of body weight). On injection, the platelets contained in 1 ml of a suspension would be redistributed in approximately 20 times the original volume. Provided that all of the platelets continued to circulate, the injection of 1 ml of a platelet suspension containing 1,000,000 per cu mm would be expected to raise the count of the recipient by 50,000 per cu mm. Table II gives the counts obtained before, and 30 minutes after, intracardiac injection of 6 platelet suspensions ("A" to "F") into 13 guinea pigs. Increases in the platelet level were observed in 9 animals and amounted to 10-80% of the calculated optimal rise. In 2 guinea pigs (No. 5 and 12) the platelet level remained unchanged and in 2 (No. 3 and 4) it dropped. Three of these guinea pigs (No. 3, 4, and 5) received a suspension ("C") which had been heated accidentally to 40°C. The 4th animal (No. 12) received a suspension ("F") which raised the platelet count of another animal (No. 13) to 80% of the optimal calculated level.

No febrile reactions to platelet transfusions occurred when sterile pyrogen free equipment and materials were used. One irradiated dog developed an extensive urticaria following the 5th of a series of transfusions.

Discussion. The tendencies of platelets to become sticky, to agglutinate rapidly, and to undergo morphologic alterations in shed blood are well known. Sequesterene Na_2 minimized these changes whereas other anticoagulants including citrate and ACD§ were ineffective. In unpublished experiments, blood decalcified by passage through a cation exchange resin column at room temperature was also found unsatisfactory for the preparation of platelet suspensions. Such blood remained fluid, but most of the platelets were retained in the column. The resins used were a sulphonic cation exchange resin, Dowex 50, and a carboxylic cation exchange resin, Amberlite IRC 50, both on the sodium cycle, buffered to pH 7.2. Amberlite IRC 50 when cooled to 1°C did not remove platelets from blood precooled to 7-9°C. Recovery of platelets from this blood, however, was not practical because of stickiness and rapid irreversible clumping. This was possibly due to the very slow flow rate at low temperatures which may have allowed changes in the platelets to occur before the blood reached the column and calcium was removed.

In the absence of adequate *in vitro* technics for determining platelet viability it has been necessary to evaluate preparations *in vivo*. A considerable proportion of the transfused platelets appeared to maintain their functional integrity in that they continued to circulate for 24-48 hours, and reversed the coagulation defect and hemorrhagic tendency in irradiated thrombopenic dogs(2). In the normal animal part of the platelet rise following transfusion might be accounted for by a rapid release of the animal's own platelets into the circulation. In the irradiated animal which produces no platelets the rise in circulating platelets can be accounted for only by the continued circu-

§ Solution of tri-sodium citrate, citric acid and dextrose, as specified in Minimum Requirements for Citrated Whole Blood, Biologics Control Laboratory, National Institutes of Health.

lation of transfused platelets. The marked variation in the rise of circulating platelets in different animals given transfusions from a single suspension, or from a different suspension prepared in identical fashion at another time, cannot be explained at present.

When complexed with calcium, Sequesterene is relatively non-toxic(3). Proescher has shown the usefulness of Sequesterene as an anticoagulant for humans(4). In our limited experience Sequesterene has been nontoxic as well as non-pyrogenic. Although large numbers of platelets were removed from the circulation of the experimental animals within 30 minutes to 2 hours of transfusion, no evidence of massive thrombosis or embolism has been found.

Summary. (1) A simple method is presented for the efficient separation and concentration of platelets from human, dog, and guinea pig blood in quantities sufficient for transfusion. (2) This method depends upon the use of Sequesterene Na_2 as an anticoagulant, which prevents the development of platelet stickiness and clumping in shed blood. (3) Platelets prepared by this method and transfused into the thrombopenic, irradiated dog remain in the circulation, reverse the coagulation defect, and prevent hemorrhage.

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γ-Aminobutyric Acid Content and Glutamic Decarboxylase Activity in Developing Mouse Brain. (19224)

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γ-Aminobutyric acid is found in the free form in large amounts in brain and spinal cord(1-4). This amino acid and the enzyme which catalyzes its formation from L-glutamic acid by alpha decarboxylation are present in uniquely high concentrations in the tissue of the central nervous system(5,6). The enzyme requires pyridoxal phosphate as a coenzyme, which can be synthesized by brain from adenosinetriphosphate and pyridoxal(5,6). When rats were fed a diet deficient in pyridoxine there was a decrease of approximately 50% in the degree of saturation of apoenzyme with coenzyme, but the content of apoenzyme was normal(7). Refeeding pyridoxine to rats previously made deficient resulted in a return of the activity to a normal level(7). In the present experiments a study was made of the content of γ-aminobutyric acid and the activity of glutamic decarboxylase in the brains of mice at various stages of development in an effort to delineate the role of this system in brain metabolism. Some correlative observations were made on the nervous tissue of cats, rats, dogs, and rabbits.

Experimental. Animals, preparation of tissues, and analyses. All mice, rats, and rabbits employed were killed by dislocation of the cervical vertebrae. The cat and dogs were killed by intravenous nembutal. Whole mouse and rat brains were used for analysis. Alcoholic extracts of the tissues were analyzed for the content of γ-aminobutyric acid by a paper chromatographic method(3) and the maximal potential glutamic decarboxylase activity was determined by a manometric pro-

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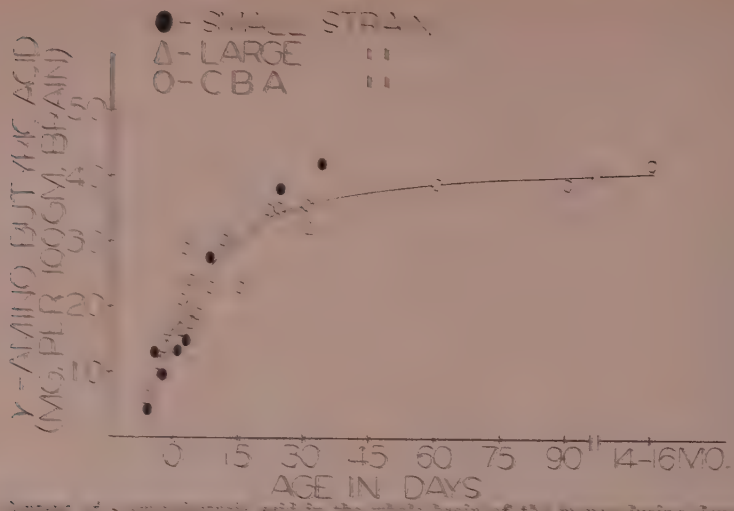

FIG. 1. Content of γ-aminobutyric acid in the whole brain of the mouse during development.

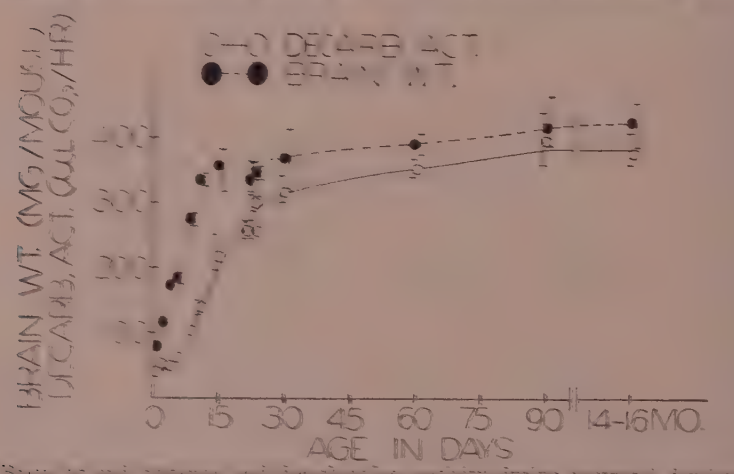

FIG. 2. [illegible]

cedure(7) using homogenates of fresh brain in the presence of an excess of pyridoxal phosphate. Analyses for γ-aminobutyric acid and the decarboxylase assays were performed on aliquots of the same homogenates prepared from several brains. Most of the mouse brains used were from animals of the CBA Jax strain. Brains from 154 mice of this strain were employed in making 49 individual determinations of both γ-aminobutyric acid and the enzyme. Each point shown in Fig. 1 and 2 is the average of from 2 to 7 determinations. Analyses for γ-aminobutyric acid were also made on 11 pooled samples of brain from Small strain mice and 10 pooled samples from the Large strain mice. The latter two strains of mice are maintained by Dr. M. Runner at the Jackson Memorial Laboratory. The rabbits used were of the New Zealand White Stock and Sandy Flemish strains. The dogs used were a 7-year-old Springer Spaniel and two 1-2-year-old Shetland Sheep dogs. The rats were of the Sprague-Dawley strain. In order to follow the histological changes which match the chemical changes observed, frozen sections in the sagittal plane at comparable

levels were cut of brains of CBA strain mice taken at postpartum intervals of 3 hours, 5, 8, 15, 19 and 22 days and 1, 2, and 3 months. These sections were stained for nucleic acids with thionin buffered to pH 5.0, for myelin by Spielmeyer's method, and for neurofibrillae by a Cajal technic. Also available for comparison were several series of other strains of mice and transverse and sagittal rat material stained for cells and fibers.

Results. γ-Aminobutyric acid content, glutamic acid decarboxylase activity, and fresh weight of developing mouse brain. All of the three variables studied increased greatly during postnatal development, attaining their maximal levels by 90 days (Fig. 1 and 2). The increases in γ-aminobutyric acid and decarboxylase are shown as increases in amounts per unit of fresh weight of tissue. The increase of decarboxylase activity lagged behind that of the weight and the γ-aminobutyric acid during the earlier period. Half-maximal values were attained at the following times: γ-aminobutyric acid, 3 days; brain weight, 6 days; glutamic decarboxylase, 15 days. At 15 days of development the γ-aminobutyric acid content and weight were 75 and 81%, respectively, of their final levels. The steadily rising concentration of γ-aminobutyric acid during the early stages of development shows that the rate of production of this amino acid by the decarboxylase exceeds the rate of its utilization during this period. The fact that a constant concentration of γ-aminobutyric acid is maintained after 60 days, when the decarboxylase activity is high, suggests that the system or systems utilizing γ-aminobutyric acid are operating at a higher level later in development. The nature of the transformations which γ-aminobutyric acid might undergo in nervous tissue is not known yet. This subject is under further investigation in the St. Louis laboratory.

Decarboxylase determinations of some samples of rat brain gave the following average values: 3 days, 41 μl CO_2; 7 days, 67 μl CO_2; 60-90 days, 290 μl CO_2. It is thus apparent that the postnatal changes in enzyme level in this species are similar to those found in the mouse.

Cytological observations. The changes occurring during postnatal development discussed in the preceding section are paralleled by drastic alterations in the brain morphology. The mouse brain at birth has largely completed the process of differentiation of neuroblast to neuron and is passing over into a period of growth and maturation of the neuronal elements themselves. Thus, in the first week it is possible to delineate all of the general cytoarchitectonic areas of the brain, although the constituent neurons are only partially differentiated and the fiber tracts substantially unmyelinated. During the second and third weeks the cell bodies increase in size, become separated from one another by markedly expanded dendritic processes, exhibit an increased prominence of Nissl granules and an apparent corresponding decrease in nuclear DNA. The axons of the major tracts become myelinated and the neuropil as revealed by silver stains grows particularly abundant. These changes occur throughout the brain but appear in a somewhat serial manner; being most noticeable first in the hindbrain, and later in the higher centers. These modifications continue, but at a diminished rate, until the end of the first month and appear to be completed within the following two months at an ever decreasing rate. On the basis of these histological observations we are led to conclude that the period of greatest increment in features which are related to maturation of the nuclear masses and fiber tracts of the CNS corresponds to the time of maximum increase in activity of the glutamic acid decarboxylase.

Correlation with previous work. A comparison of the postnatal morphological changes in the mouse with those in the rat reveals that the two species are similar. This conclusion is useful since it allows a comparison of our results with enzymatic determinations carried out in the rat(8), and the utilization of the careful quantitative measurements of Smith (9) on rat neocortex. Potter *et al.*(8), measured succinic dehydrogenase and ATP-ase activity in whole rat brains from 5 days prepartum to adult stages. The general form of curves obtained by these workers resembles that observed for glutamic acid decarboxylase in the mouse with the exception that the

changes from the first to the fourth week in rat brain represent a smoother rise and do not exhibit the spurt of activity observed during the second and third week in the brain of the mouse. Smith(9) measured cells per unit volume and total cells, volume, area, and thickness of neocortex in postnatal rats and found that the cells per unit volume diminished as the other parameters of maturation increased. Smith's curves for the quantitative changes in rat neocortex and those recorded by Peters and Flexner(10) in the guinea pig cortex are similar if the postnatal period of the rat corresponds to the prenatal period in the guinea pig; an assumption also consistent with the behavior of the two species. On this basis it should be possible to graph the enzymatic changes in whole brain of mouse and rat and in cerebral cortex of guinea pig (11) on a comparable time scale. When this is done, it is found that those enzymes which exhibit a marked increase in activity do so at a somewhat earlier maturation period in the brain as a whole than in the frontal cerebral cortex, a finding in agreement with the serial nature of morphological changes referred to above.

The activities of choline esterase(12) and carbonic anhydrase(13) in the rat, and of cytochrome oxidase, succinic dehydrogenase and ATP-ase in rat, pig, and guinea pig (8,11,14,15) all increase during the period of brain development which corresponds to the postnatal interval in the mouse. The general form of the curves for these enzymes is rather similar to that for glutamic acid decarboxylase, with the interesting exception of choline esterase. The activity of this enzyme reaches a maximum at about the same period that glutamic acid decarboxylase begins to level off, but this peak is followed by a sharp relative decrease and a secondary rise of smaller dimension. Since the highest brain centers continue to mature in the period following the climax of choline esterase activity and since histochemical localization of the enzyme indicates that it is deficient in adult dog cerebral and cerebellar cortices(16), one interpretation of this difference might be that choline esterase is more restricted in its CNS action than glutamic acid decarboxylase and the other enzymes which continue to increase in activity as long as the brain continues to enlarge and undergo differentiation.

It appears that the increase in glutamic acid decarboxylase activity is correlated with maturation of function in the CNS. Since γ-aminobutyric acid and glutamic acid decarboxylase are especially characteristic of brain and spinal cord(1-4,17) our observations lend credence to the hypothesis that the production of γ-aminobutyric acid from glutamic acid as catalyzed by glutamic acid decarboxylase is of importance in CNS activity; neuronal, interneuronal, or both.

Observations on nervous tissue of other species. A number of two-dimensional chromatograms of white and gray matter obtained by gross dissection of spinal cord of rabbits showed that there was higher concentration of γ-aminobutyric acid in the gray matter. Subsequent analyses of portions of cat brain gave the following results: gray matter (frontal tip of cortex), 64 mg % of γ-aminobutyric acid and 293 μl CO_2 per hour in the decarboxylase assay; gray matter of thalamus, 41 mg % and 326 μl CO_2 %; pooled gray matter, 43 mg % and 250 μl CO_2; pooled white matter, 28 mg % and 49 μl CO_2. These results show clearly that the levels of γ-aminobutyric acid and decarboxylase activity are higher in gray matter than in white matter. It is not known yet whether the γ-aminobutyric acid and enzymatic activity found in white matter are actually present in white matter or are a result of the presence of some gray matter in the samples examined. Determination of decarboxylase activity at different levels of rabbit spinal cord gave the following average values: lumbar, 174 μl CO_2; thoracic, 136 μl CO_2; cervical, 104 μl CO_2. Since there is a decreasing ratio of gray to white matter in going from the lumbar to the cervical regions of the spinal cord, these results are in keeping with the finding of a greater enzymatic activity in gray matter.

The failure to detect γ-aminobutyric acid in extracts of the sciatic nerve of rabbits has been reported(2). In the present study two-dimensional chromatograms were made of extracts of freshly dissected samples of cerebral cortex, optic nerve, and brachial plexus from

three dogs. γ-Aminobutyric acid was detected only in the samples of cortex. The present results are consistent with the interpretation that the γ-aminobutyric acid-glutamic decarboxylase system is only operative in the central nervous system, chiefly in the gray matter.

Summary. Brain weights, content of γ-aminobutyric acid, and the activity of glutamic acid decarboxylase were determined in brains of mice at various stages of postpartum development. All of the quantities studied increased greatly during postnatal development, attaining their maximal levels at 90 days. The increase in decarboxylase activity was slower during the first two weeks than that of the weight and the γ-aminobutyric acid. A cytological study revealed that the period of greatest increase in glutamic acid decarboxylase activity is correlated with the period of greatest increment in features which are related to maturation of the central nervous system. Examination of selected samples of nervous tissues of the cat, dog, and rabbit showed that the glutamic decarboxylase-γ-aminobutyric acid system is present chiefly in the gray matter of the central nervous system. The results of the present study were correlated with relevant data from the literature.

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Comparison of Actions of Dromoran (Nu-2206) and Morphine in Production of Vomiting.* (19225)

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Following the synthesis of 3-hydroxy-N-methyl morphinan by Schnider and Grussner (1) in the laboratories of Hoffmann-LaRoche, Inc., Randall and Lehmann(2) determined its analgesic potency in rats. They found it to be about 4 times as strong an analgesic as morphine. When doses having an equal analgesic action were given, the duration of action with Dromoran was 2 to 3 times that with morphine. In an earlier series of experiments a comparison was made of the action of Dromoran on intestinal smooth muscle with the actions of morphine, metopon, methadone, Nisentil, and meperidine(3). The intent of the present work is to compare its tendency to produce vomiting to that of morphine.

Method. Eighty-five dogs weighing between 5 and 44 kg with an average weight of 14.5 kg were used in these experiments. The animals fasted for 18-22 hours prior to each

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TABLE I. Frequency of Defecation, Nausea and Vomiting in Dogs After Subcutaneous Injections of Dromoran and Morphine.

Dromoran, mg/kg	Defecation	Nausea	Vomiting	Morphine, mg/kg	Defecation	Nausea	Vomiting
.5	8/27 (29.8%)	—	1/27 (3.7%)	2.5	4/7 (57.2%)	—	3/7 (42.9%)
1	27/39 (69.3%)	22/39 (56.4%)	1/39 (2.6%)	5	27/51 (53%)	13/19 (68.3%)	23/51 (45.1%)
2	5/11 (45.4%)	2/11 (18.2%)	0/17 (0%)	10	4/7 (57.2%)	5/7 (71.5%)	3/17 (17.7%)

injection. A total of 83 injections of Dromoran and 75 injections of morphine were made subcutaneously using concentrations of 1% and 4%, respectively. The animals were watched for not less than one-half hour after injection, at which time all of the animals were depressed except one. This dog was excited by both Dromoran and morphine. At first the animals were watched only for vomiting, but later in the experiment observations were also made on defecation and nausea, as expressed by vomiting or salivation.

Results. Only 2 animals (or 2.4%) of the entire group receiving Dromoran vomited, whereas 29 (or 38.7%) of those receiving morphine vomited. Dromoran and morphine caused defecation in 52% and 54% of the animals, respectively (Table I).

Conclusions. In doses calculated to have about the same analgesic effect Dromoran produces much less vomiting in dogs than does morphine. These doses produce defecation in equal frequency.

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Initial Effects of Pentobarbital Sodium on Water Diuresis in Dogs. (19226)

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Although experimental data have appeared in the literature concerning the effects of pentobarbital sodium and other barbiturates on water diuresis no mention has been made of the early effects of the drugs when administered rapidly in anesthetic doses. In previous studies the urine rate was determined over prolonged periods and these long collection periods masked any early response of the kidney that may have occurred. A method of recording urine rates accurately over short periods is necessary if the immediate effects of the drugs on urine output are to be studied. Such a method was employed in the present study. It has been possible, therefore, to analyze the results in terms of immediate and delayed responses.

Methods. Trained female dogs, weighing 12-14 kg, were used in these experiments. The animals had access to water up to the time of the experiment but had been without food for 24 hours. Approximately 40 minutes before recording data on urine flow the dog was given 250 cc of water by stomach tube and a short time later was loosely restrained on a dog-board in a supine position in a cool, quiet room. One ureter was catheterized according to the method described previously(1) and the urine rate was determined by observing the drops of urine issuing from the catheter and recording on a kymograph. With high urine rates the volume output was determined by measuring the urine at 2-minute intervals in a 10 cc graduate. After the diuresis was

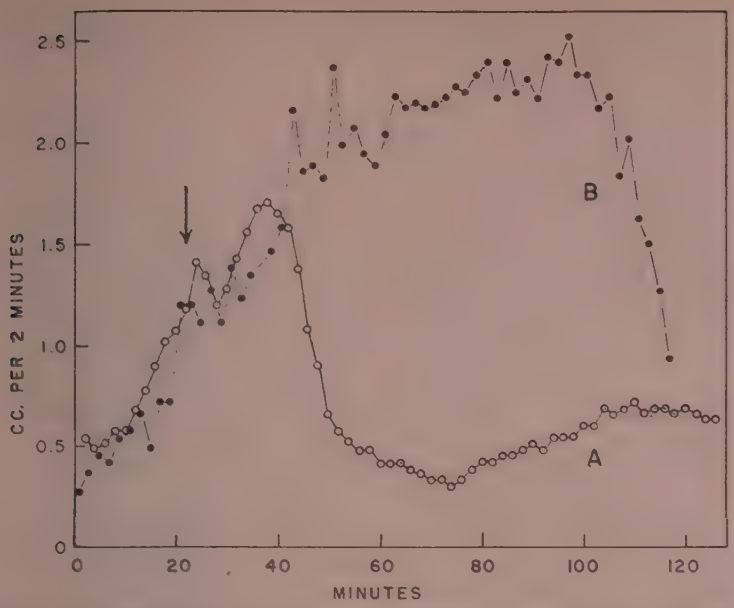

FIG. 1. Effect of pentobarbital sodium on water diuresis in dog. Curve A: Animal received 27 mg of anesthetic per kg intravenously at arrow. Curve B: Water diuresis in same dog without anesthesia. Animal received 250 cc water by stomach tube in both experiments at minus 37 min. Each point on curve is urine output from one kidney for preceding 2 min.

well-established pentobarbital sodium (Nembutal, Abbott) in a dose of .27 mg per kg body weight was administered intravenously within a period of 10 seconds. At no time during the injection was the determination of the urine rate interrupted. The recording continued until the animal showed definite signs of recovering consciousness. Because both kidneys normally respond alike to water diuresis, a response verified in these same dogs earlier (1), only one ureter was catheterized and the urine rate from one kidney determined. By alternating the ureter catheterized in a series of experiments it was possible to use a dog more often because with less frequent catheterization trauma to a ureter was minimized.

Results. A total of 24 experiments were carried out on 5 dogs, 20 studying the effects of the anesthetic on water diuresis and four controls without anesthesia. Curve A of Fig. 1 presents the data from a representative experiment. Shortly after the anesthetic was injected an inhibition of the diuresis occurred. This initial inhibition was temporary and gave way to a continuation of the diuresis. A second and prolonged inhibition occurred which gradually disappeared as the dog recovered consciousness. Fourteen of the 20 experiments with anesthesia showed the initial temporary antidiuresis which was followed by the prolonged antidiuresis. In the remaining 6 experiments the animals responded with the prolonged antidiuresis only. This delayed and prolonged inhibition of diuresis was so pronounced in 8 of the experiments that no recovery of urine rate was noted even though the recording continued in some experiments for 160 minutes after the anesthetic was administered.

Curve B represents the diuretic response in the same dog to an equal volume of water but without anesthesia. Although the urine rate showed fluctuations the diuresis lasted for more than 120 minutes without any definite temporary or permanent inhibition. Considering the fact that the animal was unanesthetized during the entire period and that the rate was determined every 2 minutes, the urine output followed a fairly smooth curve, a response which was the general rule provided

the animal remain quiet and undisturbed.

In 4 experiments on 2 dogs in this series blood pressure was recorded with a mercury manometer by direct femoral arterial puncture before, during and for a short time after the injection of the anesthetic. No urine rates were determined in these experiments. A precipitous fall in pressure of from 20 to 40 mm occurred immediately after each injection but the hypotension periods did not persist longer than 20 minutes. This temporary hypotension is the response generally seen when anesthetic doses of pentobarbital sodium are given quickly by vein.

Discussion. That anesthetic doses of barbiturates generally inhibit water diuresis in animals has been reported previously. But the mechanism of this inhibition is still a debatable issue. From the review of the literature by deBodo and Prescott(2) it appears as if the usual response of the water diuretic dog to anesthetic doses of barbiturates is an inhibition of the diuresis. From their own experiments deBodo and Prescott(2) reported that phenobarbital sodium given intravenously to water diuretic dogs in doses of 40, 80, and 110 mg per kg inhibited the diuresis in every normal dog studied; but when amytal sodium or pentobarbital sodium was given, even in full anesthetic doses, the inhibition of water diuresis occurred in only some of the dogs. The antidiuretic effect of the barbiturates was not present in dogs with the entire neurohypophysis destroyed and which showed a permanent diabetes insipidus. Silvette(3) reported that anesthetic doses of pentothal sodium inhibited the diuresis resulting from intraperitoneal injections of 0.2% NaCl solution in normal and hypophysectomized rats. He attributed the antidiuretic effect to the hypotension produced by the anesthetic and not to any increased secretion of the antidiuretic hormone (ADH). deBodo and Prescott(2) contend that in Silvette's animals not all of the neurohypophysis was removed because no permanent polyuria occurred in the animals and an increased secretion of ADH could still take place with anesthesia.

Just how the increased secretion of ADH during anesthesia occurs is a moot point. Fee(4) postulated that in the normal animal there is a mechanism which inhibits the secretion of the ADH and that anesthesia removes this inhibition and brings about an increased secretion of the hormone. Pickford(5) concludes that the inhibition of water diuresis in most cases of anesthesia is due, in part, to an increased secretion of the ADH.

Because of the rapidity with which the diuresis was initially and temporarily inhibited, and with the simultaneous fall in blood pressure, it is probable that the first antidiuresis in the present work is the result of a hypotension as suggested by Silvette(3). Although only 4 blood pressure determinations were made in these trained animals the hypotension which results from quick intravenous injections of pentobarbital sodium has been verified repeatedly in laboratory animals. The delayed and more prolonged antidiuresis can be attributed to an increased secretion of the ADH since it is present even though the general systemic blood pressure is at or near normal levels.

Summary. The intravenous injection of an anesthetic dose of pentobarbital sodium into 5 trained dogs showing a water diuresis produced a quick temporary inhibition of the diuresis in 14 of 20 experiments. The urinary rate recovered and approached the diuretic rate but a second and more prolonged inhibition of the diuresis occurred. The remaining 6 of the 20 experiments showed an immediate and prolonged inhibition of the diuresis. In 12 of the 20 experiments the urinary rate at the time the experiment was concluded was beginning to recover. The immediate temporary antidiuresis could be correlated in time of onset and duration, with a period of hypotension that followed each dose of anesthetic. The delayed prolonged antidiuresis was not correlated with hypotension and is believed to be due to increased liberation of antidiuretic hormone.

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Various Antibiotics and 3-Nitro-4-Hydroxyphenyl Arsonic Acid in Corn-Peanut Meal Rations for Swine.* (19227)

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Jukes *et al.*(1) reported a marked growth-promoting effect from aureomycin when fed in a corn-peanut meal ration. Later Cunha *et al.*(2) published the results of 12 experiments with corn-peanut meal rations in which aureomycin supplementation approximately doubled the growth rate of pigs. Other investigators(3-5) have demonstrated the value of aureomycin when fed with other type rations. Brown and Luther(6) and Carpenter (7) have reported that terramycin was equally as effective as aureomycin in promoting growth. Carpenter(7) also reported that chloromycetin increased gains but failed to control diarrhea as did penicillin, streptomycin, aureomycin, and terramycin. A growth stimulating effect from 3-nitro-4-hydroxyphenyl arsonic acid has been noted by Carpenter(8).

This study was undertaken to determine the value of adding terramycin, chloromycetin, bacitracin, and the arsonic acid derivative to a corn-peanut meal basal ration. Two levels of aureomycin were also compared.

Experimental. The animals used in the experiment were purebred Duroc, Hampshire, and Spotted Poland China. They were allotted carefully according to breed, sex, and weight and placed on experiment at approximately 9 weeks of age. The basal ration fed to all groups consisted of yellow corn 57%, peanut meal (hydraulic) 41.5, bone meal 0.5, limestone 1.0, and a salt-trace mineral mixture 0.53 (iodized salt 50 lb, manganese sulfate 921 g, ferrous sulfate 398 g, copper sulfate 125 g and cobalt carbonate 10 g). Crystalline V-vitamins were added at the following levels per 100 lb of ration: thiamine 1 g, riboflavin 230 mg, niacin 2.33 g, pantothenic acid 1 g, pyridoxine 375 mg, choline 19.4 g, folic acid 22.7 mg, and vit. B_{12} 1 mg. Vit. A and D were administered by capsule at weekly intervals. The animals were self-fed on concrete floors which were washed down daily.

Results. Effects of the various treatments are summarized in Table I. The two levels of aureomycin (lots 2 and 3) and terramycin (lot 4) resulted in an increase in gains over the control lot and all other treatments which was statistically significant at the 5% level. The difference in gains between the two levels of aureomycin was not statistically significant

TABLE I. Effect of Antibiotics and 3-Nitro-4-Hydroxyphenyl Arsonic Acid on Growth of Swine.

Lot No.	1	2	3	4	5	6	7
Supplements to basal (per 100 lb ration)	Basal	Basal+1 g aureomycin*	Basal+2 g aureomycin*	Basal+2 g terramycin†	Basal+ 2 g chloromycetin‡	Basal+2 g bacitracin§	Basal +1.7 g 3-nitro-4-hydroxyphenyl arsonic acid‖
No. of pigs	6	6	6	6	6	6	6
Days on test	70	70	70	70	70	70	70
Avg initial wt, lb	31.7	31.6	31.6	31.7	31.6	31.7	31.7
" final " "	75.1	106.7	120.6	114.1	77.9	65.3	86.9
" daily gain "	.62	1.07¶	1.27¶	1.18¶	.66	.48	.77
" daily feed "	1.95	3.35	3.47	3.46	2.57	2.26	1.98
Feed/lb gain "	3.07	3.33	2.73	2.94	3.92	4.70	2.51

* Supplied by Dr. T. H. Jukes, Lederle Laboratories, Pearl River, N. Y.
† Supplied by Dr. H. G. Luther, Chas. Pfizer and Co., Brooklyn, N. Y.
‡ Supplied by Dr. O. D. Bird, Parke, Davis and Co., Detroit, Mich.
§ Supplied by Dr. M. A. Schooley, Merck and Co., Rahway, N. J.
‖ Supplied by Dr. T. W. Zbornick, Dr. Salsbury's Laboratories, Charles City, Iowa.
¶ Difference from basal statistically significant at 5% level.

* This study was supported in part by grants from the National Vitamin Foundation, and the Lasdon Foundation. Florida Agric. Exp. Station Journal Series, No. 32.

FIG. 1. A pig showing the dermatitis syndrome, observed in all lots except 2 and 3.

not were the gains of pigs receiving aureomycin significantly different from those of pigs receiving terramycin. Chloromycetin failed to stimulate growth. This is particularly interesting in view of the fact that chloromycetin is a "wide spectrum" antibiotic similar in scope to aureomycin and terramycin. The arsenic acid derivative did not cause a significant increase in gains but did result in feed efficiency considerably better than for all other lots. Aureomycin and terramycin stimulated appetite as indicated by daily feed consumption figures, which probably accounted in part for the large gains.

Occasional scouring occurred during the first four weeks of the experiment in lots 1, 5, 6, and 7. Scours were most severe and continued longest on the bacitracin (lot 6). Two pigs in each of lots 1, 5, and 6 showed a dermatitis syndrome. One pig each in lots 4 and 7 was afflicted with this condition. All of these animals gained very poorly during the experiment. A typical pig is shown in Fig. 1.

Summary. Of the various substances tested in this experiment only aureomycin and terramycin gave significant growth response. This response was approximately the same in magnitude. These two antibiotics controlled an intermittent type of diarrhea observed on the basal ration. The other treatments were not effective in this respect.

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Observations of 3-Hydroxy-N-Methylmorphinan Hydrobromide (Dromoran®) on Fetal Respiratory Movements of the Rabbit.* (22825)

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3-Hydroxy-N-methylmorphinan hydrobromide is a synthetic analgesic chemically related in structure to morphine marketed under the registered name Dromoran®. Clinical reports(1-3) indicate that it is 3 to 4 times as potent as morphine and that certain side effects common to morphine are minimal or absent. It has not been advocated as an obstetrical analgesic but its chemical relationship to morphine as well as the importance of any analgesic to the obstetrician has led to an investigation of the effect of Dromoran®

* This study was aided by a grant from funds granted through Initiative 171, State of Washington. The Dromoran was supplied by Hoffmann-La Roche, Inc., Nutley, N. J.

TABLE I. Effect of Dromoran Administered Intravenously Into Maternal Circulation on Respiratory Rate of the Mother and on Mean Respiratory Rate of the Fetuses.

Dose, mg/kg	Respiratory rate	Mean respiratory rate at end of: Control	5 min	15 min	30 min	60 min	90 min	2 hr	3 hr	4 hr
1.5	M*	200	56	50	46	40		38	42	50
	MF	15.5	6.5	1.5	10.2	15.3		26.3	16.9	22.6
2	M	306	50		27		27	30	43	48
	MF	14.1	.3		2.4		3.4	6.2	2.4	4.2
3	M	145	55		55		74	80	85	82
	MF	13.9	0		.36		10.7	8.9	8.8	.36
4	M	360	51		38	46	41	40	42	
	MF	14.7	1.1		[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	
4.5	M	150		15	20	19	31	45	59	85
	MF	35		1.3	1.2	1.2	.9	4.6	8.1	8.4
4.5	M	90	48	45	100	127		144	68	65
	MF	11.6	0	0	.25	1		2.7	3.3	1.7
5	M	315	26	23			45	43	Maternal death	
	MF	11.6	0	6.5			2.2	.8	in 145 min	
5	M	143	3	30		34		33	Maternal death	
	MF	14.5	0	0		0		0	in 135 min	
5	M	360		20	25	37		34	44	150
	MF	14.9		0	1.5	7.6		14.9	13.1	5.5
10	M	270	18	16		13	13	16	18	26
	MF	13.6	0	0		0	0	0	[illegible]	[illegible]
15	M	110	32	37	27	30	24	26	46	53
	MF	1.6	0	0	0	0	0	0	0	[illegible]
20	M	280	11	37	35	23		13	14	23
	MF	17.7	0	0	0	0		0	1.6	6.8

* M = maternal. MF = mean fetal.

on the fetal respiratory movements of the rabbit.

Methods. Snyder(4) has reported that regular rhythmic respiratory movements occur in fetuses during a period of immediately before birth and has shown that these movements are influenced by drugs administered to the maternal circulation. The procedures used were modified from those reported by Snyder and Rosenfeld(5) and Bonar and Blumenfeld(6). Approximately 7 days before the expected termination of pregnancy, a dose of 120 to 130 IU of an extract of pregnancy urine (Antuitrin S®, Parke-Davis) was administered intravenously. This hormone inhibits uterine contractions and minimizes this effect on the respiratory activity of the fetus (7). Within a day or two of the expected delivery a spinal transection was carried out under local anesthesia at the level of T10 or T11. The rabbit was then placed in a large bath containing Ringer solution maintained at 37°C so that the lower trunk was submerged. By means of midline incision the uterus was exposed and opened permitting the fetuses to rest lightly on a small supporting shelf under the surface of the bath. Maternal respirations were recorded by means of a chest pneumograph and the fetal respiratory movements were recorded by means of a series of keys operating signal magnets. After a control period the Dromoran® was administered by the marginal ear vein of the mother in such a concentration as to make a total volume of approximately 2 cc.

Results. Table I gives a summary of the effect of doses of 1.5 mg to 20 mg per kg of Dromoran® administered to the mother on maternal respiration rate and on the mean fetal respiration rate.

Considerable variation was observed in the fetal respiratory movements of a single rabbit. This can be seen in Table II which presents the complete data from an experiment in which the mother was given 4.5 mg per kg. This table indicates that while there was considerable variation between individual fetuses all showed decreased respiratory activity from the administration of this dose to the mother.

Discussion and summary. Dromoran®

TABLE II. Effect of Intravenous Administration of 4.5 mg Dromoran® per kg to the Maternal Circulation on Respiratory Rates of the Mother and Fetuses.

4.5 mg/kg	Control	15 min	30 min	60 min	90 min	2 hr	3 hr	4 hr
Maternal	150	15	20	19	31	45	59	85
Fetus: 1	24	0	0	0	0	0	14	10
2	30	4	6	6	4.5	14	18	15
3	26	0	0	0	0	2	2	7
4	31	0	0	0	0	5	5	0
5	65	2.5	0	0	0	2	1.5	10
Mean fetal rates	35	1.5	1.2	1.2	.9	4.6	5.1	8.4

passes without difficulty across the placental barrier and produces a decrease in the rate of respiratory movements of the fetuses. This passage across the placental barrier might be expected in view of the general principle stated by Needham(8) that the placental barrier is freely passed by practically all substances of low molecular weight. Thus in obstetrical analgesia one can assume that the agent will pass the placental barrier and the best analgesic will be one which will have high analgesic potency in the mother and a minimal effect on fetal respiration activity. The final evaluation of an analgesic must therefore be made under clinical conditions where the analgesic property can be evaluated in the patient. The doses of Dromoran® used in these experiments were much higher than would be used for human patients and there is no doubt but what satisfactory analgesia in humans can be obtained with lower doses. The question of whether these doses depress the respiratory function of the baby can be answered by clinical observation only with difficulty. On the basis of the parallelism shown in the rabbit experiments between the response of the maternal respiration rate and the rate of fetal respiratory movement it would appear safe to assume that if, in the human mother, respiratory function was depressed there would almost certainly be a corresponding effect on the respiratory function of the baby. Thus an obstetrician might make use of changes in the maternal respiration rate as an index to the respiratory function of the baby.

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Dibenamine Blockade as a Method of Distinguishing Between Inotropic Actions of Epinephrine and Digitalis.*†

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A differentiation of the inotropic effects of epinephrine and digitalis has direct application in the characterization of such drugs as the veratrine alkaloids. These alkaloids produce cardiac actions which resemble, in some particulars, both those of epinephrine and

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† Preliminary abstract in *Fed. Proc.*, 1951, v10, 289.

digitalis(1). The present report describes conditions under which Dibenamine blocks about 70% of the inotropic action of epinephrine without significantly affecting the inotropic response of digitalis. The pharmacology of Dibenamine has been comprehensively reviewed by Nickerson(2).

Methods and Procedure. The contractile force of a segment of the right ventricle was determined with strain gauge equipment attached to modified Cushny levers in open-chest dog preparations. These preparations routinely included the use of barbital-pentobarbital anesthesia and bilateral cervical vago-sympathectomy. Twenty-eight such experiments were conducted. Additionally, 2 experiments were conducted in intact-chest preparations using a more compact type of strain gauge equipment stitched to the anterior aspect of the right ventricle on the previous day. The conditions and limitations of these methods have been previously described(3,4) and their use in characterizing various drug groups has been described recently(5,6). Electrocardiographic recordings of standard lead II were made in most experiments with a direct writing EPL Cardiotron. N,N-dibenzyl-b-chloroethylamine (Dibenamine) in the form of its hydrochloride salt was administered in doses of 20 or 40 mg/kg by intravenous infusion over a 20 minute period. Fresh 1% solutions in distilled water were prepared each day. Freshly diluted epinephrine solutions were made every 20 to 30 minutes from the stock 1:1000 solution of the hydrochloride (Adrenalin, Parke-Davis). Test doses of epinephrine were 3 γ/kg in the open-chest preparations and 2 γ/kg in the intact-chest preparations. Digitalis was administered in the form of the diluted tincture by intravenous infusion in doses of 0.75 cat unit/kg over intervals of 30 minutes. Similarly, in 2 experiments, a potent extract of erythrophleum alkaloids was used in doses of 0.5 cat unit/kg.

In 8 experiments, digitalis was administered after Dibenamine had abolished the major portion of the inotropic response to epinephrine. In 4 other experiments, digitalis was administered after Dibenamine under similar conditions of dose and time interval but without such tests of epinephrine blockade. Another series of 14 experiments was designed to determine the conditions of the Dibenamine-produced blockade of epinephrine inotropic responses. These included determinations of the influence of post-Dibenamine epinephrine hypotension; marked reductions in body temperature; responses to multiple dose injections of epinephrine following Dibenamine; and effects of repeated epinephrine injections over intervals of 4 to 5 hours without Dibenamine. These 14 experiments were conducted in open-chest preparations with controlled body temperatures. In the case of multiple dose injections of epinephrine following Dibenamine, 2 additional experiments were conducted in intact-chest preparations. In 5 of these experiments with epinephrine, the contractile force and blood pressures were recorded simultaneously with a Brush BL-902A oscillograph. Pressures from the carotid artery were recorded in these instances by means of a Statham transducer amplified through a Brush Analyzer Model BL-310. Arterial hypotension was counteracted by means of a ligature placed around the thoracic aorta and drawn out through a glass tube in the posterior chest wall. Body temperatures were maintained at approximately constant level by means of electrical heat pads. In all cases, body temperatures were determined by mercury bulb thermometers inserted about 18 cm beyond the anal orifice to about the level of the sigmoid colon. In 2 special experiments, in which the body temperatures were deliberately altered, reduction was accomplished by placing crushed ice on the body surface; temperatures were subsequently raised by means of electrical heat pads.

Results. The positive inotropic action produced by epinephrine was substantially reduced by the prior administration of Dibenamine. The experiment shown in Fig. 1 demonstrates that the administration of 3 γ/kg of epinephrine 37 minutes after completing the Dibenamine administration resulted in a reduction of the inotropic response by about 85%. Reversal of the hypertensive response to epinephrine was obtained within 21 minutes after Dibenamine administration. In 20 other experiments, approximately similar degrees of

FIG. 1. Myocardiograph and arterial pressure tracing. Upper tracing obtained with Cushny levers typically attached to the anterior aspect of the right ventricle in the open chest, vagotomized dog preparation under barbiturate anesthesia. Encircled figures represent contractile force in g determined by means of strain gauge equipment attached to Cushny levers. Drugs administered intravenously. Time marker in min. Dibenamine (20 mg/kg) administered between first and second sections. Fifteen min elapsed between completion of Dibenamine infusion and beginning of the second section of tracing.

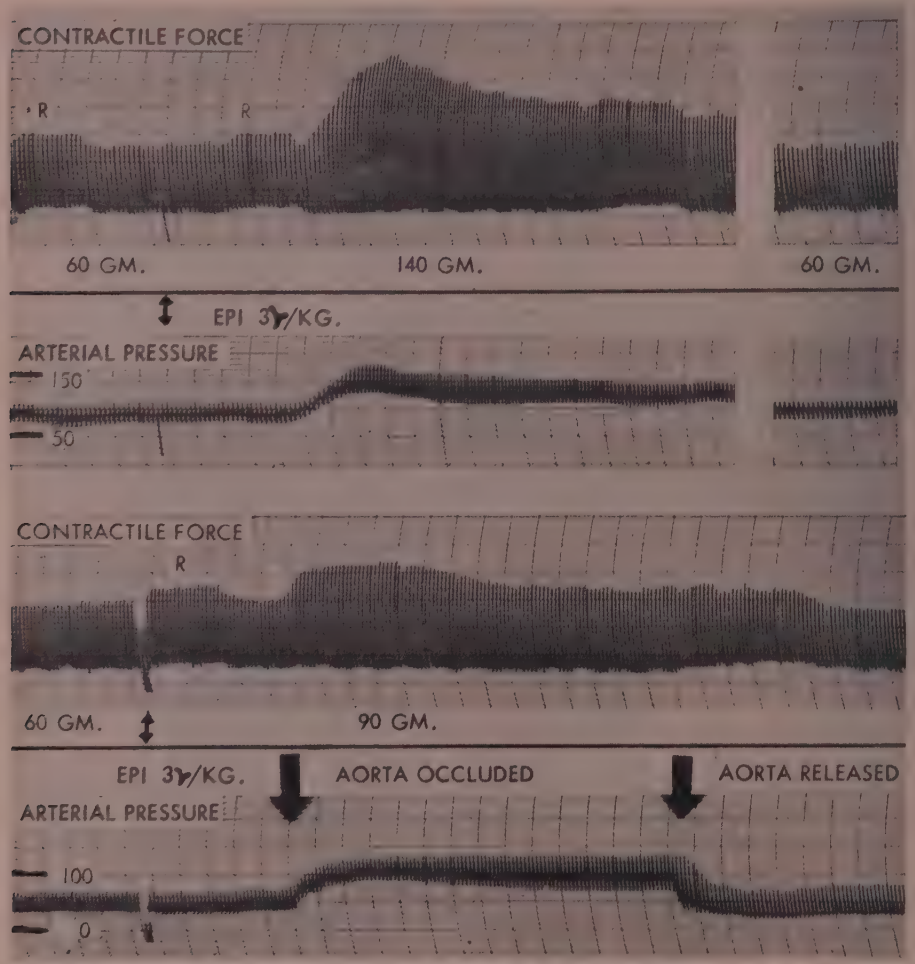

FIG. 2. Synchronous oscillograph recordings of contractile force and arterial pressure. In upper pair of tracings the injection of epinephrine increased contractile force by 133%. The lower pair of tracings were obtained from the same preparation 107 min after Dibenamine had been infused in a dose of 40 mg/kg. In this case the same dose of epinephrine increased contractile force by only 50%. The letter R designates intervals of inspiratory movements which, at times, have a limited but recognizable effect on contractile force; calculation of contractile force is based on intervals between such respiratory movements.

epinephrine inotropic blockade were obtained. The average reduction of the inotropic response for all these 21 experiments was about 70% of the control response. The maximum reduction (about 90%) was obtained in 6 of the 21 experiments. During each individual experiment, the blockade reached its maximum in 35 to 110 minutes and then did not change in any important degree. Blockade was approximately as complete with doses of 20 mg/kg as with doses of 40 mg/kg Dibenamine. Inotropic responses to multiple dose injections of epinephrine after Dibenamine were studied in 1 open-chest and in 2 closed-chest experiments. In these cases, the epinephrine inotropic response to single test doses of 2 or 3 γ/kg was reduced by about 75% after Dibenamine. When multiple test doses (up to 8 single doses) were given after Dibenamine, the positive inotropic response was usually less than that obtained by one single test dose in the control period. In all of these calculations, the inotropic response was evaluated in terms of the percent change from the immediately preceding control period.

Effect of repeated epinephrine injections without Dibenamine administration. In 2 experiments, with stabilized body temperatures but without Dibenamine administration, 10 injections of 3 γ/kg of epinephrine over intervals of 4 to 5 hours did not result in substantial differences in the quantitative inotropic responses. These time intervals under parallel conditions were longer than the intervals over which the experiments were conducted with Dibenamine.

General effects of Dibenamine. Characteristically under these conditions, Dibenamine produced lowered body temperature, hypotension, tachycardia and, in about half of the instances, a substantial increase in contractile force. The question naturally arises as to whether the blockade just described is secondary to some of these changes or whether it is a local, specific cardiac action. The following described observations demonstrate that the effect is direct and specific. At the same time, they serve to estimate the influence of these different variables on the final quantitative values.

Effect of body temperature on the Dibenamine blockade of epinephrine inotropic responses. The effect of a marked decrease and subsequent increase in body temperature on the quantitative inotropic response produced by epinephrine was examined in 2 experiments without Dibenamine administration. Frequent injections of 3 γ/kg were given as the temperature was altered. A reduction of the body temperature to 30°C from a control of 37°C did not appreciably reduce the quantitative inotropic response to epinephrine. This reduction of temperature did not significantly change the contractile force of the heart during the interval between the injections. Similarly, increasing the temperature from 30°C to 38°C did not appreciably influence the inotropic response to epinephrine or the control level of contractile force. On the other hand, the chronotropic response to epinephrine was markedly altered under these same conditions. As the temperature fell, the peak rates produced by epinephrine were drastically reduced (from 200/min. to 110/min.). The control rates were also reduced but were less markedly affected (from 155 min. to 100/min.). When the body temperature was raised from 30°C to 38°C, opposite effects of approximately the same magnitude were observed. The results of these experiments, in which the body temperature was markedly altered, are considered to demonstrate the relative stability of the contractile force measurements over this subnormal range. Other studies(7) have demonstrated markedly increased contractile force during advance stages of hyperpyrexia. In 3 open-chest experiments, in which the body temperature was maintained by heat pads within 0.5°C of the control (38°C), the inotropic responses to 3 γ/kg of epinephrine were blocked to the same degree as was produced in the other epinephrine-Dibenamine experiments without temperature control. In all of these 3 experiments the contractile force during the control intervals was increased moderately and the heart rate markedly (up to 240/min.) following Dibenamine administration. When body temperatures were not artificially maintained, heart rate increases were less pronounced and the chronotropic responses to epinephrine were considerably diminished. These experiments,

in which the body temperature was either altered or kept constant demonstrated that the blockade of the inotropic response of epinephrine by Dibenamine was not directly related to or dependent upon the level of the body temperature.

Circulatory changes produced by Dibenamine. In about half of 26 experiments, the administration of Dibenamine produced an increase in contractile force of approximately 55%; in 10 experiments there was no important change and in 2 there was reduction of contractile force by about 25%. In those experiments with contractile force increase, the greatest portion of the effect was established by the time of completion of the Dibenamine infusion; it was persistent and was not related to changes in blood pressure or heart size. This change was associated with an average increase in heart rate of about 35 beats per minute and usually a slow, progressive fall in blood pressure. Blood pressure usually stabilized at about 70-80 mm Hg but in some cases reached levels of 40 mm Hg. Electrocardiographic changes were usually limited to a sinus tachycardia and moderate S-T segment depression with variable T-wave changes. In those experiments without important contractile force changes due to Dibenamine, the increment in heart rate and the decrease in blood pressure were less pronounced. The blockade of epinephrine inotropic response was not related to these variabilities in blood pressure, heart rate or contractile force levels.

Possible influence of epinephrine produced hypotension on inotropic blockade. In the immediately preceding experiments, it was observed that Dibenamine-produced blockade of epinephrine inotropic responses occurred as fully at high blood pressure levels as at lower levels. It is well known that in the absence of Dibenamine the epinephrine inotropic response is readily elicited at levels of profound hypotension or in instances of complete circulatory failure. Nevertheless, there is reason to consider the possibility that the immediate hypotension produced by epinephrine following Dibenamine may have an influence on the quantitative inotropic response. The fact that such hypotension is not obtained with digitalis would suggest that this might be the basis for the distinction between their inotropic blockade characteristics. The experimental observations of this study, however, fully demonstrate that this immediate hypotensive phase is of little consequence in the production of epinephrine inotropic blockade. For instance, it was seen that the maximal contractile force increments typically occurred before the full hypotensive action was developed. In 5 experiments with synchronous oscillograph recordings of arterial pressure and contractile force, it was determined that the maximal contractile force response to epinephrine typically developed before the pressure had fallen more than 25% of its full hypotensive phase. In several other experiments, arterial pressure was maintained at or above control levels by tensing a ligature previously placed around the thoracic aorta; in such cases there was no significant difference in the degree of inotropic blockade as compared with similar experiments in which hypotension developed in the usual way (Fig. 2). Further, in some instances, in which blood pressure had reached levels of 40 mm Hg there was no hypotensive phase in the epinephrine response and in such cases there was the usual grade of blockade of inotropic responses.

Effect of Dibenamine on the inotropic action of digitalis. Typical increments in contractile force were obtained with digitalis in 8 experiments after the demonstrated blockade by Dibenamine of about 75% of the epinephrine inotropic response. The experiment shown in Fig. 1 demonstrates that, following blockade of about 85% of the contractile force response to epinephrine, the inotropic response to digitalis was 43% of the control level. As compared with a previous characterization study with the digitalis glycosides(3), this represented no important reduction of the usual inotropic response to digitalis. The absence of any important digitalis inotropic blockade in all of the 8 experiments was demonstrated by a quantitative comparison with the previous study with measured inotropic responses to digitalis. Such quantitative comparisons include recognition of the previously demonstrated variation in responses obtained at varying control levels. The characteristic

rise in blood pressure ordinarily seen with digitalis also occurred in these experiments. In 4 of the 8 experiments, in which the blood pressure was above 90 mm Hg before digitalis administration, the average increase in arterial pressure was 41 mm Hg as compared with 43 mm Hg average increase obtained in the previous digitalis study. The average increase in blood pressure for the other 4 experiments was only 10 mm Hg; in these latter experiments, the blood pressure before digitalis administration was about 55 mm Hg. The failure to obtain a typical rise in blood pressure in these 4 experiments was probably related to special features of the hypotension produced by Dibenamine. The associated electrocardiographic changes seen in the digitalis-Dibenamine experiments were typical of those obtained following digitalis administration. Typical inotropic increments were also produced by the erythrophleum alkaloids in 2 experiments in which there was demonstrated blockade of the epinephrine inotropic response. The erythrophleum alkaloids had previously been shown to produce characteristic digitalis-like effects on the mammalian heart (8,9).

In 2 experiments, without demonstrated epinephrine inotropic blockade but following similar Dibenamine administration and time intervals, digitalis produced typical contractile force increments.

In 2 additional experiments, in which an extreme hypotension (30 mm Hg or less) developed after Dibenamine, the subsequent administration of digitalis was followed by progressive depression of contractile force and blood pressure with termination of the experiment. These depressant effects were clearly related to the generally deteriorated condition of the preparations and they illustrate a limitation in the application of these procedures.

Discussion. Previous reports, based chiefly on experiments with heart-lung and papillary muscle preparations, have shown that Dibenamine under such conditions does not alter the inotropic effect produced by epinephrine (10,11). The present experiments, on the contrary, involve the complete organism, large doses of Dibenamine and relatively considerable intervals of time. Under such conditions a substantial degree of blockade is demonstrable.

Summary and conclusions. In open-chest, vagotomized dogs, Dibenamine has been shown to be effective in producing a substantial blockade of the inotropic effects of epinephrine while leaving the inotropic effects of digitalis relatively unaffected. This inotropic blockade of epinephrine by Dibenamine is not the result of secondary cardiovascular changes.

We are obliged to Smith, Kline and French Co. for their supplies of Dibenamine.

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Hormonal Influences on Epinephrine Induced Aortic Sclerosis. (19230)

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Josue(1) produced, experimentally, degenerative aortic lesions in rabbits as an effect of repeated epinephrine injections. Since this original observation, a voluminous literature on this subject has accumulated. An extensive summary of this work is presented by Hueper(2). The incidence of the production of aortic lesions varies from author to author, both in the number of animals which are affected and in the intensity of the sclerosis in an individual animal. This variation ranges from 10% up to 100%. Although many attempts have been made to influence the course of epinephrine-induced sclerosis, our survey of the literature does not reveal any report on the influence of thyroxin and desoxycorticosterone on this process. It is noted, however, that Lortat and Sabareanu(3) found that thyroidectomy prevents the epinephrine-induced sclerosis. Others, however, could not confirm this. Theoretically, it would be expected that thyroxin should enhance the sclerosis induced by epinephrine. This is based on the well known observation that the sensitivity of tissue to epinephrine is increased by thyroxin. Specifically, it has been shown that epinephrine elicits a much greater blood pressure rise in dogs fed with thyroxin as compared to the normal control dogs. This augmentation of epinephrine effect by thyroxin could not be demonstrated on the vessels of the isolated limbs of dogs by Mikulicich(4). Desoxycorticosterone is reported to produce a vasopressor action with a general blood pressure rise (5). In addition, Selye and Stone(6) found desoxycorticosterone in large repeated doses resulted in sclerotic changes in the arterioles of chicks and rats. Arteriosclerotic changes also have been obtained from repeated implantations of adrenal cortex(7).

Methods. Fifty-seven rabbits were used in our study. The different groups were injected daily with the following materials: (1) epinephrine, intravenously, 50 μg of freshly made solution per kg, 1:1000 U.S.P. ampule diluted with sterile 0.9% sodium chloride solution. Some of the animals were given 25 μg per kg for the first 2 days, as a precaution against initial fatality. (2) Thyroxin, subcutaneously, 0.25 mg per kg, prepared from a 1 mg per ml ampule or prepared from thyroxin crystals* in a sterile 0.9% sodium chloride solution containing 2 drops of 0.4% sodium hydroxide. (3) Desoxycorticosterone acetate (cortate†), subcutaneously, 0.5 mg per kg and 1.0 mg per kg, of a 5 mg per ml solution in sesame oil. (4) Combinations of these.

Results. Table I outlines the results obtained. Animals which died after the first to fifth injection of epinephrine are grouped as early deaths. These deaths occurred within one-half hour after the intravenous administration of epinephrine, in a typical epinephrine asphyxial death, several animals showing hemorrhagic foamy sputum; such early deaths are not included in the observations for sclerosis. The one early death in the thyroxin group was a markedly cachectic animal, the death apparently being due to cardiovascular failure. All of the animals treated with thyroxin showed a rapid loss of weight and pronounced tachycardia. Aortic sclerosis was considered slight if only a few small plaques were seen in the supravalvular or thoracic aorta. Sclerosis was considered medium when numerous plaques were seen along the entire course of the aorta, some of which were confluent, partly ulcerated, and or calcified. Severe sclerosis was recorded when whole segments of the aorta were involved, including the lower abdominal aorta.

Epinephrine alone produced only slight or medium sclerosis. Thyroxin alone produced no sclerotic changes, in spite of general cachexia. A combination of thyroxin and epinephrine showed sclerotic changes in all 8

* Thyroxine kindly supplied by E. R. Squibb and Co.

† Cortate supplied through courtesy of Schering, Inc.

TABLE I. Aortic Sclerosis Associated with Hormone Injections.

Daily treatment	No. of rabbits	Early deaths	Gross aortic sclerosis				Later deaths	Sacrificed after 26 days
			None	Slight	Medium	Severe		
E* (50)	14	4	2	4	4	0	4	6
T† (.25)	5	1	4	0	0	0	2	2
E (50) T (.25)	14	6	0	1	3	4	8	0
E (50) DCA (.5)	2	0	2	0	0	0	0	2
E (75) DCA (.5)	2	0	1	0	1	0	0	2
E (50) DCA (1)	13	3	9	1	0	0	4	6
E (50) T (.25) DCA (1)	7	4	0	0	0	3	3	0
Totals	57	18	18	6	8	7	21	18

* Epinephrine (E): in μg per kilo, intravenously.
† Thyroxine (T), and desoxycorticosterone acetate (DCA): in mg per kilo, subcutaneously.

rabbits so injected, most of the animals demonstrating a more severe grade of aortic sclerosis than was seen in the epinephrine group. In some of the cases where the animals died after only five injections, this severe degree of sclerosis was already present. In the animals treated with epinephrine and desoxycorticosterone only a slight degree of sclerosis was observed in 2 out of 14 animals. In the rabbits treated with a combination of epinephrine, desoxycorticosterone, and thyroxin, all 3 of the 3 animals showed a severe degree of sclerosis, which occurred very early, as soon as 6 days after beginning the treatment.

Prominent changes were observed in the appearance of the lungs of the animals which died, during the first 4 days of treatment, immediately after epinephrine injection. The lungs of animals treated with thyroxin and epinephrine showed a marked pulmonary edema and were severely hemorrhagic, their appearance resembling the red hepatization of pneumonitis. Animals which received only epinephrine also showed marked edema, but no diffuse hemorrhage, only localized petechiae. Animals treated with desoxycorticosterone and epinephrine showed pulmonary edema, which was almost without hemorrhage. However, this latter group in general showed varying degrees of clear serous ascites, with edematous engorgement of some tissues. Detailed pathological studies will be reported later in a separate communication.

Discussion. The induction of sclerotic lesions in arteries, produced by excessive dosage of epinephrine, has been attributed to two possible mechanisms. One of these is the effect of anoxemia on the vessel walls as a result of the severe vasoconstriction. As a part of this action, the blood flow in the vasa-vasorum may be indirectly affected. The second mechanism is that of an effect of epinephrine on the cellular metabolism, especially the biochemical chain of oxidative catalysis. Study of the concentration of some of the oxidative enzymes in human aortas, cytochrome C, and alloxazine-adenine dinucleotide and phosphopyridine dinucleotide (coenzyme I and II), showed a marked decrease of these enzymes in sclerotic aortas which was roughly proportional to the degree of sclerosis(8). It is known that thyroxin enhances the degree of vasoconstriction caused by epinephrine, and also increases the oxygen consumption of tissue generally; in the light of this, the high and severe incidence of sclerotic lesions in rabbits treated with both thyroxin and epinephrine becomes more understandable. The stress on the cardiovascular system, as a result of this combined treatment, further increases the oxygen need of the tissues involved. This cardiovascular stress was evidenced in the permanent tachycardia seen after several days of epinephrine and thyroxin treatment. In many cases, the normal heart rate of approximately 200 per minute was increased to as high as

360 per minute.

The almost complete absence of sclerotic changes in the group of animals treated with epinephrine and desoxycorticosterone is difficult to explain. Further, the Selye group(9), working on chicks and rats, obtained hypertension and vascular changes with the use of desoxycorticosterone. The well known action of desoxycorticosterone in the regulation of cell membrane permeability and its effect on electrolyte balance may be involved in facilitating detoxication in a state of hypoxic metabolism. When thyroxin is added to this treatment desoxycorticosterone failed completely to inhibit the sclerotic changes. It is possible that the permeability changes due to desoxycorticosterone are no longer sufficient to compensate for the enormous catabolic injury to the tissues of the vessels.

Summary. Thyroxin and epinephrine treatment in rabbits was shown to produce aortic sclerosis in a greater percentage of cases, and a more severe grade of such sclerosis, than epinephrine alone. Desoxycorticosterone decreases the amount of sclerosis due to epinephrine alone but failed to influence the sclerosis due to the combined epinephrine-thyroxin treatment.

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Effect of Cortisone Administration upon Nucleic Acid Composition of Rabbit Liver.* (19231)

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Though purines appear in increased amounts in urine of both animals and humans treated with cortisone or ACTH(1-4), it is not clear whether the uricaciduria is merely a reflection of decreased tubular reabsorption of uric acid(1,4), or results partly from lymphoid tissue destruction, since this atrophies following the administration of ACTH(5-7). A portion of the pentose nucleic acid (PNA) of hepatic cells of animals is labile and can be reduced by chemical injury(8,9) and starvation(9-11), and returns readily during recovery from either type of stress. Because of these observations, experiments were planned to demonstrate whether cortisone could reduce the amount of PNA in the liver.

Plan of experiments. Cortisone was injected intramuscularly in daily doses of 25 mg for 1, 2, 3, and 6 days in 10 albino rabbits, 1-2 kg in weight. The animals were sacrificed 24 hours after the last dose of cortisone. In addition, 2 animals received cortisone for 3 days and were sacrificed 9 days later. Six animals served as controls. The animals were stunned by a blow on the head, then exsanguinated by severing the hepatic vein. The livers were dissected free and weighed after removal of the gallbladder. A 2-4 g portion of the liver was weighed wet, then dried first over a water bath, then in an oven at 100°C to constant weight. A second portion (2-4 g)

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TABLE I. Effect of Parenteral Administration of Cortisone on Composition of Rabbit Livers.

Animal No.	Days cortisone	% dry wt	mg N/g wet wt	μg DNA/g wet wt	μg PNA/g wet wt	PNA/DNA
59	0	—	30.60	616	2685	4.36
				645	2830	4.39
1	0	31.34	35.70	807	2692	3.34
				789	2847	3.61
2	0	29.44	35.70	786	2987	3.80
				821	3163	3.85
3	0	31.64	38.10	817	3543	4.34
				884	3899	4.41
4	0	32.80	36.55	764	3495	4.57
				772	3418	4.43
203	0	29.50	29.07	541	2670	4.94
				569	2871	5.05
Avg & S.D.	0	30.94 ± .38	34.21 ± 4	734 ± 108.42	3091 ± 394.8	4.26 ± 1.49
99	1	30.90	22.93	382	2056	5.38
				401	1956	4.88
100	1	29.58	24.15	486	2098	4.32
				513	2058	4.01
Avg	1	30.24	23.54	446	2042	4.65
101	2	31.05	21.40	284	1830	6.44
				287	1680	5.85
102	2	32.09	20.12	356	2306	6.47
				356	2130	5.98
Avg	2	31.57	20.76	321	1987	6.19
97	3	30.98	20.70	294	2148	7.31
				305	1990	6.52
98	3	31.21	16.30	227	1556	6.85
				227	1734	7.64
199	3	28.50	17.75	300	2631	8.77
				300	2676	8.92
200	3	29.40	20.50	396	2600	6.57
				358	2485	6.94
Avg	3	30.02	18.81	301	2228	7.44
43	6		20.30	310	2489	8.03
				323	2571	7.96
44	6		17.00	231	2103	9.10
				257	1972	7.67
Avg	6		18.70	280	2284	8.19
201*	3	27.90	23.40	438	2390	5.52
				433	2537	5.86
202*	3	26.30	31.20	564	3514	6.23
				498	2463	4.95
Avg	3	27.10	27.80	482	2726	5.64

* These animals received cortisone for 3 days but were not killed for 9 days thereafter.

was expressed through a meat press onto a torsion balance pan, weighed to .01 g and then quantitatively transferred to a Potter-Elvehjem glass homogenizer. The homogenate was diluted with slightly alkaline 0.85% NaCl so that each ml of solution contained 0.1 g of wet liver and portions thereof were used for all chemical analyses. Two 2 ml fractions of the brei were used for nucleic acid analyses. This was thoroughly mixed with 5 ml of trichloroacetic acid (TCA), kept at 10°C for one hour, centrifuged, and the precipitate washed once with 5 ml of 5% TCA. The supernatants were pooled and subsequently analyzed for DNA (desoxypentose nucleic acid) and PNA. Since under the conditions of the experiments there was no difference in the supernatants in the values for DNA and PNA between the controls and the cortisone-injected animals, these results will not be tabulated. The sediment was extracted with 5 ml of 5% TCA at 80°C for 30 minutes, centrifuged, re-extracted once more with 5 ml of 5% TCA for 5 minutes at 80°C, and recen-

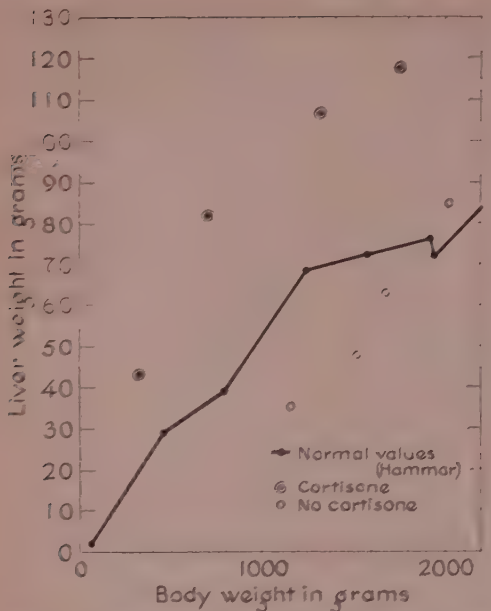

FIG. 1. Relationship between body wt and liver wt. Wt for 4 normal and 4 cortisone treated animals from the present experiment are recorded. Also included are normal values obtained from Hammar(21) from a much larger series of rabbits.

trifuged. These supernatants were pooled and analyzed for DNA and PNA in duplicate. PNA was determined by the orcinol reaction; DNA by diphenylamine color; and nitrogen by a micro Kjeldahl procedure in duplicate on 1 cc of 1:10 brei. These have been described previously(12). One ml of concentrated HCl was added to 9 ml of the original brei. This was then placed in boiling water for 10 minutes. After centrifuging, reducing substance in the supernatant was determined by the Somogyi method(13,14). When kidneys were analyzed for PNA, DNA, and N, they were treated in the same fashion as the livers. Tissues for histological examination were obtained immediately after the whole liver was weighed. These were placed in Lavdowsky's solution(15), and then examined with the following stains: Feulgen (DNA)(16), Ribonuclease-Toluidine blue (PNA)(16,17), periodic acid, Schiff's (glycogen)(18), Ponceau-2R (acidophilic protein)(19), Sudan III (lipid), and Hematoxylin-Eosin.

Results. Table I summarizes the days of cortisone administration, changes in wet and dry weight, nitrogen, PNA,† DNA,† and the ratio PNA/DNA. The following may be noted: The per cent of dry weight did not change appreciably, even though total liver weight was apparently increased (Fig. 1). The total N per g of wet weight decreased steadily with increasing time of cortisone administration. This was also true of the DNA. The concentration of PNA decreased only for the first two days and then remained almost the same thereafter. Because of the difference between the behavior of the DNA and PNA, the ratio of PNA/DNA became progressively greater. The livers of the two animals that received cortisone for 3 days only and were subsequently sacrificed 9 days later, showed results which were intermediate between those of the normal animals and those treated for 3 days, except for the per cent dry weight. This had decreased appreciably, suggesting some edema of the liver.

Table II indicates that there was a considerable increase in reducing substance, presumably glycogen, in the liver of the animals in which this was determined.

The nucleic acid composition of the kidney was determined in 4 animals before the administration of cortisone and in 2 animals after administration of cortisone for 3 days. In the untreated animals the average value of PNA was 1670 μg/g and for DNA 1246 μg/g, ratio PNA/DNA being 1.34. After cortisone the PNA was 1715 μg/g and DNA 1131 μg/g, while the ratio was 1.51 Composition of the kidney was not significantly altered by this type of treatment. In the 2 animals that had received cortisone, the livers were analyzed and the characteristic alterations anticipated were observed. It is obvious, therefore, that while the liver is significantly affected by cortisone administration, the kidney is not.

The histochemical observations are summarized in Table III and illustrated in Fig. 2 to 5. They confirm the chemical observa-

† The notations DNA and PNA when used in the text refer to total nucleic acid while in the tables indicate only the carbohydrate moieties, since these were the components measured. mg Desoxyribose x 2.3 = mg DNA. mg Ribose x 2.14 = mg PNA.

TABLE II. Carbohydrate Content of Rabbit Liver.

	Animal wt, kg	Liver wt, g	% CHO in liver	Total CHO in liver, g	$\frac{\text{Liver CHO}}{\text{Body wt}} \times 1000$
No cortisone	2.08	84.5	2.09	1.77	.85
	1.18	36	3.25	1.17	.992
Cortisone 3 days	1.26	107.5	5.32	5.72	4.54
	1.76	118	4.65	5.49	3.12
	.750	83	3.04	2.52	3.36

TABLE III. Histochemical Examination of Rabbit Livers.

	Normal	Cortisone	Cortisone-recovery
Acidophylic protein (Ponceau 2-R)	4*	1*	3*
Glycogen (PA-S)	2*	4*	3*
PNA (toluidine blue pH 7)	4*	1*	3*
DNA (Feulgen)	1*	1*	1*
Sudanophilic lipid	1*	1*	1*

* The intensity of staining has been graded from 1 to 4*.

tions and further indicate that during the relatively short period of cortisone administration there was no striking increase in fat in the liver.

Discussion. Though sufficient data are lacking to select the proper explanation for changes in liver composition, several of these deserve mention. A first possibility is as follows: Under the influence of cortisone two phenomena occurred. (1) liver cells increased in size (as the microscopic sections indicate) and (2) there was an absolute increase in the amount of PNA. These two effects provided (a) that the increase in cell size was relatively greater than the increase in PNA, and (b) that the DNA remained constant per cell, would decrease per g of liver of the PNA and raise the ratio of PNA/DNA. There is evidence for and against this explanation. That there could have been a two-fold increase in the PNA seems doubtful from the evidence furnished by Skipper *et al.*(20). They observed that cortisone actually inhibited the incorporation of C^{14} labeled formate by both DNA and PNA in the viscera of mice. Hence, synthesis of new PNA under the present experimental conditions seems unlikely. That there was an absolute increase in the size of the livers of the cortisone-treated rabbits is suggested by Fig. 1. No evidence can be advanced to indicate that liver weights had increased sufficiently to provide the observed alteration in the concentration of PNA and DNA, for it was obviously impossible to weigh the livers before and after cortisone. But even if such data were available these could not explain the change in the ratio PNA/DNA. Because of these objections, this explanation for the observed changes cannot be entirely acceptable.

The second explanation which can be advanced is that there was a decrease in both PNA and DNA per cell, but the decrease in DNA was greater than that of PNA. To attempt to support this hypothesis, information was sought from histochemical observations. Evidence was required as to whether PNA actually had increased or decreased in each cell. Examination of the sections stained with toluidine blue indicates that in the cortisone-treated animals, there was a tremendous decrease in the ribonuclease-hydrolyzable basophilia (PNA) per cell (Fig. 3, 5). Since the PNA seemed to decrease in each cell, the alterations in PNA/DNA ratio could only have occurred if there had been a concomitant decrease in the DNA which was greater in amount than the decrease in PNA. It has always been assumed that DNA-histone when once formed was insoluble in the living cell (22), only cell death resulting in its removal. Were this correct, then the results indicated by this experiment would be at least somewhat anomalous. This is particularly true since there was no evidence of cell necrosis following cortisone administration though serial sectioning of the livers was not performed. How-

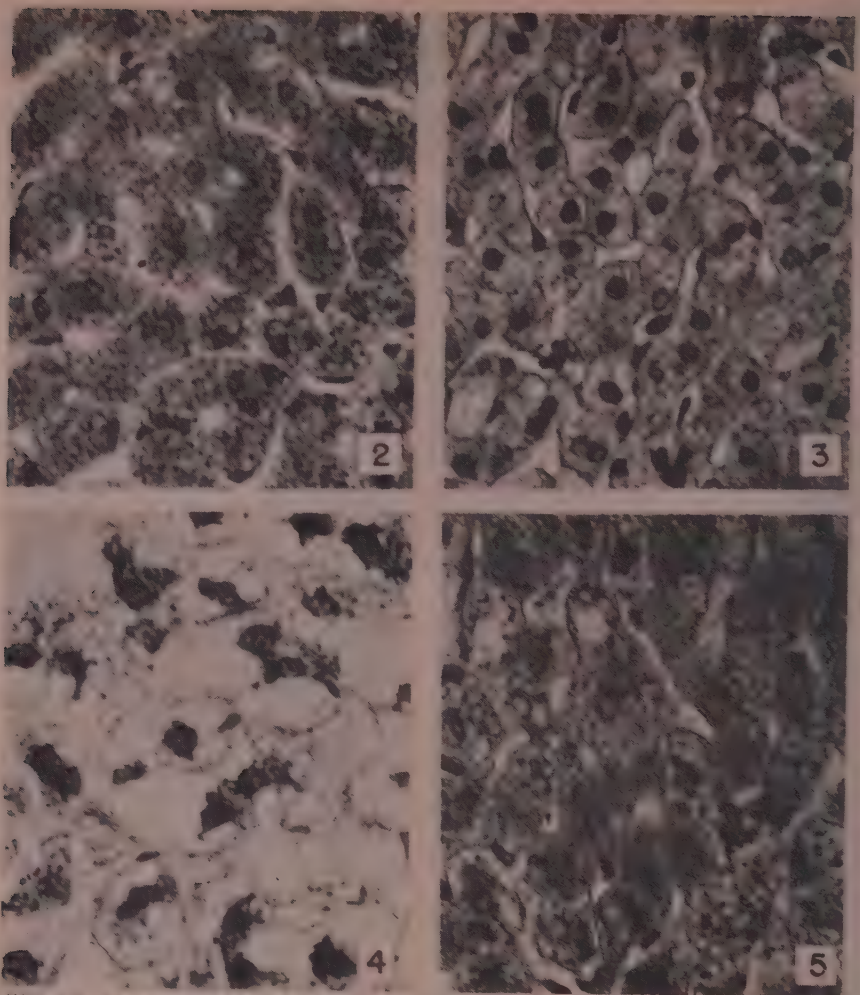

FIG. 2-5. 10 μ sections of rabbit livers stained with .06% toluidine blue at pH 7 for 25 min. Sections in Fig. 2, 4 and 5 were hydrolyzed in Clark-Lubs' buffer, pH 6.8 at 37°C for 5 hr prior to staining. Section in Fig. 4 was hydrolyzed under identical conditions in buffer containing 1 mg of ribonuclease per ml. 500 ×.

FIG. 2. Normal rabbit. Note the abundant, granular cytoplasmic basophilia.

FIG. 3. Section of same liver as above. Absence of granular cytoplasmic basophilia following ribonuclease hydrolysis.

FIG. 4. Six days of cortisone administration (animal 43). Cytoplasmic enlargement, and absence of basophilia except in perinuclear zone.

FIG. 5. Cortisone administration for 3 days followed by 9 days' "recovery" (animal 201). Note partial repletion of cytoplasmic basophilia.

ever, Germuth *et al.*(23) have reported that necrosis does occur in the livers of rabbits treated for 18 days with 10 mg of cortisone per day. Schneider and Hogeboom suggest that at least 60% of the DNA in the resting liver cell nucleus is soluble(24). Furthermore, Putnam and Kouloff(25) from studies of bacteriophage production by bacteria suggested that only 30% of the bacterial DNA was genetically important. The remainder might be the soluble DNA of Schneider and Hogeboom, and also that portion which was released from the livers of rabbits to whom cortisone had been administered. This second

explanation depends for its justification largely upon the staining characteristics of PNA. It is possible that under the altered conditions which obtain in a liver cell following the administration of large doses of cortisone, there would be interference with the normal affinity of PNA for toluidine blue. Therefore, this explanation also cannot be accepted without reservation.

A third explanation might be advanced. The change in concentration of DNA might be due to destruction of nonhepatic cells, particularly lymphocytes. The disappearance of these cells in conjunction with an increase in hepatic cell size would yield analytical results of the sort obtained. This concept would receive substantial support if it were known that lymphocyte cytoplasm contained less PNA per cell than did liver cells, and if it were possible to demonstrate a significant difference in the number of lymphocytes between the normal and cortisone-treated animals. A careful search of the normal rabbit livers failed to demonstrate any significant number of lymphocytes, hence cortisone treatment could produce no decrease. However, serial sections would be necessary to establish this point beyond question.

It is known that cortisone causes enhancement of infection by certain bacteria(26) and viruses(27). Although there was no evidence of infection in the rabbits used in these experiments, the possibility that the biochemical alteration in the liver may have been due to injury by an undetected infectious agent cannot be excluded. A partial solution to the dilemma posed by these observations may come from the technic of counting nuclei and determining the quantity of DNA and PNA per nucleus. By this means it should be possible to ascertain whether these actually are altered in each living cell and, if so, the extent of this change.

Summary and conclusions. Livers of rabbits that received 25 mg of cortisone for 1, 2, 3, and 6 days were studied chemically and histologically. Under this stimulus, nitrogen content decreased and carbohydrate content increased in the livers. The PNA and DNA per g of liver both decreased but the ratio of PNA/DNA rose steadily with increasing number of days of cortisone administration. Analysis of livers of 2 animals killed 9 days after cessation of cortisone injection indicated repletion of DNA, PNA, and nitrogen. The nucleic acid composition of the kidneys was not altered by cortisone. A number of alternate explanations for the observations are advanced.

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Antithyroxine Properties of *N*-(2,5 Dihydroxyphenyl) Pyridinium Acetate.* (19232)

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(Introduced by Joseph L. Lilienthal, Jr.)

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With the increasing evidence that the circulating thyroid hormone is free thyroxine(1-3) an added importance is afforded recent studies (4-6) directed at determining agents which may possibly inhibit the action of thyroxine. Such research may elucidate the little understood mechanisms whereby the thyroid hormone acts to stimulate the metabolism of body cells as well as to regulate thyrotropin production by the anterior hypophysis. As Harington(7) has indicated, structural analogues of thyroxine possessing properties metabolically competitive to thyroxine are of physiological interest and potential clinical application. The observations by Woolley(4) that several structural analogues of thyroxine inhibit thyroxine-induced metamorphosis of tadpoles have been confirmed and extended by Williams *et al.*(8), by Frieden and Winzler (9,10), and in our laboratory(11). However, several investigators(8,11-13) have been unable to demonstrate that these analogues inhibit to any noteworthy degree the effect of thyroxine when tested in animals of higher forms.

In 1948, while the tadpole metamorphosis test was being used as a screening method to determine the antithyroxine potentialities of a large number of compounds, it was found in this laboratory that *n*-(2,5-dihydroxyphenyl) pyridinium acetate† (DHPPA) appeared to have such properties. Subsequently, the effects of this compound on the oxygen consumption of normal rats and of normal rats receiving thyroxine were observed. These studies comprise this report.

Methods and results. A. *Metamorphosis of tadpoles. Xenopus laevis* tadpoles, bred and raised according to the method of Deansley and Parkes(14), were kept in "livered" tap water‡ and fed a mixture of dried powdered liver and flour until they reached 18-26 mm in length. The tadpoles were transferred to "livered" water in beakers of 250 ml capacity, 5 tadpoles to each beaker; graded doses of thyroxine and/or DHPPA were added, and the final volume of each beaker was brought up to 200 ml. All beakers were placed in a water bath at room temperature. At the end of the third day the tadpoles in each beaker, after a brief rinsing to remove any residual drug, were transferred into clean beakers, each containing 200 ml of "livered" water. The beakers were returned to the water bath until the seventh day when the test was terminated. The animals received no food during the experimental period. Each tadpole was observed daily for evidence of metamorphosis.

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† Kindly supplied by Dr. Elmer Sevringhaus, Hoffmann-La Roche, Nutley, N. J.

‡ "Livered" tap water was prepared by adding 100 mg dried powdered liver to each gallon of tap water and then filtering after 24 hours. This procedure removes excess chlorine.

TABLE I. Effect of *n*-(2,5-dihydroxyphenyl) Pyridinium Acetate (DHPPA), With and Without *dl*-thyroxine, on the Metamorphosis of *Xenopus Laevis* Tadpoles.

	Thyroxine*	DHPPA*	No. of tadpoles	Tadpoles positive on day: 4 No.	4 %	5 No.	5 %	6 No.	6 %	7 No.	7 %
A.	.05	—	14	2	14	12	86	14	100	14	100
		.5	10	0	0	0	0	0	0	0	0
		5	10	0	0	0	0	0	0	0	0
	.05	.5	9	0	0	6	66	8	89	9	100
	.05	5	9	0	0	0	0	0	0	5	55
B.	.05	—	14	11	79	12	86	14	100	14	100
		5	15	0	0	0	0	0	0	0	0
		20	15	0	0	0	0	0	0	0	0
	.05	5	14	2	14	7	50	9	64	10	71
	.05	20	15	0	0	3	20	4	27	5	33

In A tadpoles varied from 20 to 26 mm in body length; temp. averaged 22.2°C (range 20-23.5), and pH of bathing medium at termination of experiment varied from 5.5 to 5.7. In B tadpole length varied from 18 to 22 mm; temp. averaged 24°C (range 23-24.6), and pH of medium at termination of experiment varied from 6 to 6.5. pH was determined with nitrazine paper. Twenty control tadpoles receiving no drugs during each experiment did not show metamorphosis.

* Dosage of thyroxine and DHPPA is expressed in mg per 100 ml environmental water.

A response was considered as "positive for metamorphosis" when one or both forelimb buds erupted through the skin. In preliminary experiments 0.05 mg % *dl*-thyroxine in the tadpoles' environmental water for 3 days uniformly produced metamorphosis of all tadpoles within 7 days, and was not lethal. Since the rate of metamorphosis was affected by environmental temperature, control groups receiving only thyroxine were run with each experiment. Animals receiving no drugs never showed metamorphosis during the experimental periods. The tadpole offspring from separate matings of different *Xenopus* frogs were used in 2 successive experiments. Results are shown in Table I. An antagonism to thyroxine apparently proportional to the concentration of DHPPA was noted. DHPPA appeared to be non-toxic to tadpoles in the concentrations used. In a later experiment involving the use of 40 tadpoles it was found that concentrations of DHPPA, 35.0 or 50.0 mg %, with or without *dl*-thyroxine, 0.05 mg %, were lethal to the tadpoles within 48 hours.

B. *Oxygen consumption of rats.* Eight male Sprague-Dawley rats, weighing about 100 g each, were maintained on Purina dog chow and water. Over a period of 34 days the oxygen consumption of each animal was determined at intervals of 4 to 8 days; this served to condition the animals to the apparatus. The animals were fasted 16 to 24 hours prior to testing. The oxygen consumption of each animal was determined in a closed circuit volumeter at constant temperature and pressure. The original apparatus described by Lilienthal, Zierler and Folk(15) was used. When the 8 rats attained a weight of about 250 g each, they were divided into 4 groups of 2 animals each, and the experiment recorded in Table II A was conducted. The values of oxygen consumption represent the average of the 2 animals in each group. A progressive decrease of basal metabolism of growing rats is well recognized(16), and was noted in these 8 rats in the preliminary period. The rather marked reduction in oxygen consumption seen in Table II A was probably due to the fact that these animals were still in the growth phase. The marked decrease of oxygen consumption by the control animals 4 days after injection may also have been due to the fact that on the day prior to this determination the weather was exceptionally hot and humid. The results show that DHPPA did not significantly alter oxygen consumption nor prevent the rise induced by thyroxine.

To determine if time and mode of administration of DHPPA were important, an additional experiment was conducted as recorded in Table II B. Animals of Groups II, III, and IV were employed, using the final de-

TABLE II. Effect of [illegible] Dihydroxyphenyl) Pyrrolidinium Acetate (DHPPA), With and Without Thyroxine, on the Oxygen Consumption of Adult Rats.

			Oxygen consumption, c.m.m. × min. / g body wt							
			Before drug			After administration of drug				
	Group		15 days	8 days	Avg	1 day	% change*	4 days	% change*	13 days	% change*
A	I	Saline—2 c.c./kg s.c.	1.84	1.78	1.81	1.74	— 3.9	1.67	—13.8	—	—
	II	Thyroxine—2 mg/kg s.c.	1.71	1.71	1.71	1.82	+11	1.64	— 7.2	1.53	—10.4
	III	DHPPA—200 " "	1.97	1.94	1.96	1.91	— 4	1.88	— 4.9	1.40	—12.1
	IV	Thyroxine—2 " "	1.62	1.58	1.60	1.80	+12.4	1.52	— 4.8	1.41	—11.9
		DHPPA—200 " "									
						1 day	% change	4 days	% change		
B	V	Thyroxine—2 mg/kg s.c.			1.76	1.92	+28.9	1.59	—2.8		
		DHPPA—200 " "									
	VI	DHPPA—200 mg/kg i.p.‡ (immediately before thyroxine)			1.42	1.66	+17.7	1.46	—1.4		
		Thyroxine—2 mg/kg s.c.									
	VII	DHPPA—200 mg/kg i.p. (24 hr before thyroxine)			1.45	1.78	+29.8	1.89	—4.1		
		DHPPA—200 mg/kg i.p. (immediately before thyroxine)									
		Thyroxine—2 mg/kg s.c.									

* Change in % as compared with avg reading before drug.
† s.c. = subcutaneously. ‡ i.p. = intraperitoneally.
In Groups IV and V injections were made at separate sites.

termination of oxygen consumption of the first experiment as the base-line oxygen consumption of the second experiment. Again, DHPPA did not prevent the increase in oxygen consumption induced by thyroxine. The differences in the magnitude of the increases are considered of no significance.

Discussion. These experiments indicate that DHPPA inhibits thyroxine-induced metamorphosis of tadpoles but fails to prevent an increase in oxygen consumption of rats given thyroxine. However, certain major differences in the experiments are apparent. In the tadpole experiments the thyroxine and DHPPA were mixed together in the animals' environmental water, whereas injections of these materials were made at separate sites in the rats. The possibility that DHPPA may alter thyroxine thereby diminishing or destroying its physiological activity must be considered. The compound may possibly alter the absorption of thyroxine by the tadpoles. Since DHPPA in the concentrations used did not appear toxic to the tadpoles, it does not seem likely that the failure of metamorphosis was due to the tadpoles' inability to respond to thyroxine. Finally, the discrepancy of results in the two experiments may have been due to inadequate dosage of DHPPA in the rats.

Barker *et al.*(12) have observed the effects of this compound on the oxygen consumption of rats. DHPPA 9.5 mg/kg/day, for 6 days did not alter significantly the oxygen consumption of normal rats, and a sharp increase of oxygen consumption occurred following the injection of thyroxine. However, in rats receiving DHPPA, 95 mg/kg/day, there was, respectively, a 10.5 and 12.8% drop in oxygen consumption after 8 and 13 days of drug administration, but in these groups response to thyroxine was not tested. They also observed that in thyroidectomized rats receiving thyroxine, 12 μg/kg/day, no depression of oxygen consumption was produced when DHPPA was given at 100 and 500 times the molar concentration of thyroxine for 15 and 28 days, respectively. While these investigators did not demonstrate that DHPPA inhibited the action of administered thyroxine, they did observe that large doses of DHPPA reduced the oxygen consumption of normal rats. In these latter experiments the total doses of DHPPA were approximately 4 to 6 times greater than those used in our experiments.

Conclusions. (1) *N*-(2,5-dihydroxyphenyl)

pyridinium acetate inhibited the metamorphosis of tadpoles induced by thyroxine. The degree of inhibition of metamorphosis appeared proportional to the relative concentration of the drug. (2) *N*-(2,5-dihydroxyphenyl) pyridinium acetate, 200 mg kg body weight, did not lower the rate of oxygen consumption of normal rats nor prevent the acceleration of oxygen consumption induced by *dl*-thyroxine, 2 mg kg body weight.

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The Potentiating Effect of Alcohol on Thiopental Induced Sleep.* (19233)

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Studies of the action of thiopental on rats (1) show that alcohol tolerant animals are much more resistant to thiopental than are normal rats. The induction time of alcohol anesthesia in mice is significantly reduced when the animals are premedicated with a barbiturate(2). Observations of a different nature show that the sleeping time of thiopental treated animals is greatly prolonged by premedication with Antabus(3). However no evidence is available concerning the effect of alcohol on thiopental sleeping time. The present report is concerned with the effect of alcohol in subanesthetic doses on the duration of sleep produced by thiopental. Attempts were made to determine an analgesic effect of alcohol and the influence of thiopental on alcohol analgesia.

Methods and materials. Initially an attempt was made to determine the effect of thiopental, alcohol and combined thiopental-alcohol administrations on the response of mice to a painful stimulus. The apparatus described by Chen and Beckman(4) was used for determining analgesic effect. This consisted of a metal chamber which was open to the atmosphere only through a reflux condensor. The chamber was partly filled with an azeotropic mixture of ethyl formate and acetone which has a boiling point of 55°. The chamber was heated with a hot plate so that the azeotropic mixture boiled continuously. The mice were dropped on the top of this container 2 minutes after intravenous injection of the drugs. The time required for the animal to respond by licking its hind foot was determined with a stop watch and was recorded as the reaction time.

Observations of the duration of sleep produced by the intravenous administration of thiopental, alcohol, and combined thiopental-alcohol were made. The thiopental dosage

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TABLE I. Effect of Alcohol, Thiopental and Alcohol Plus Thiopental on the Reaction Time in Mice.

Animal group	No. of animals	Drug	Dose	Reaction time, sec	Stand. dev.	Probability of diff.*
A Control	59	0		9.92	± 3.17	
B "	10	.09% saline sol.	1 ml	6.91	± 2.71	Comparing A and B, P = .10
C Test	9	Thiopental	5 mg/kg	4.01	± .80	B C, <.001
D "	10	Alcohol	1000 "	7.6	± 2.19	B D, >.50
E "	10	Alcohol+ thiopental	1000 " 5 "	5.94	± 1.95	B E, >.20

* Probability according to Student "T" test.

TABLE II. Effect of Alcohol, Thiopental and Alcohol Plus Thiopental on Sleeping Time of Mice, Rabbits and Dogs.

Animal Group*	Drug	Dose (mg/kg)	Mean sleeping time (min)	Stand. dev.	Probability of diff.†
Mouse	Thiopental	30	6.7	± 2.78	P = <.001
	Alcohol	1000	.5	± .2	
	Thiopental + Alcohol	30 + 1000	32.7	± 8.6	
Rabbit	Thiopental	25	21.4	± 2.7	P = <.001
	Alcohol	1000	12.5	± 4.5	
	Thiopental + Alcohol	25 + 1000	117	±28.6	
Dog	Thiopental	20	38.8	±15.6	P = .001
	Alcohol	1000	0		
	Thiopental + Alcohol	20 + 1000	71.5	±18.5	

* 10 animals in each group.
† Probability according to the Student "T" test.

was: Mice—30 mg/kg; Rabbits—25 mg/kg; Dogs—20 mg/kg. All animals received 1000 mg/kg alcohol as a 10% solution in isotonic saline. The combined thiopental-alcohol dosage contained these same amounts of the drugs. Sleeping time was designated as the time during which the righting reflex was absent. The righting reflex was recorded as being present when the animal would voluntarily roll to its side when placed on its back. Blood levels of alcohol were determined in the rabbits and dogs using the technic of Hemingway, Bernat and Mashmeyer (5).

Results. Table I indicates that intravenous thiopental in a subanesthetic dose in mice caused them to react to the painful stimulus more quickly than did the control animals in which nothing was injected, or in which only saline was injected. When alcohol was injected in a subanesthetic dose there was no significant change in the reaction time to pain. Larger doses were not used because they caused loss of consciousness, therefore making it impossible for the animal to give the characteristic sign that it was experiencing pain. When both alcohol and thiopental were given simultaneously, the mice did not show a significant change in their reaction time to the painful stimulus (Table I).

Table II presents that data obtained in experiments to determine the sleeping time induced by alcohol and thiopental injected separately and together in mice, rabbits and dogs. In all animals a significant increased duration of sleep is induced by administration of both alcohol and thiopental when compared with either compound administered alone. In these experiments whole blood showed maximum concentrations of alcohol in the range of 150 to 200 mg % in all animals used in the study.

Discussion. The foregoing data indicate that the sleeping time induced by thiopental is significantly prolonged by the simultaneous administration of alcohol. Giarman *et al.* (3) noted that the thiopental sleeping time could be prolonged by pretreatment with

Antabus. Previously Richert(6) had concluded that Antabus, which blocks the oxidation of alcohol at the acetaldehyde stage, inhibits the oxidation action of xanthine oxidase. Giarman's data led to the inference that xanthine oxidase is one enzyme involved in the metabolism of thiopental. Therefore, one explanation of the prolongation of the thiopental induced sleeping time by alcohol may be that the two compounds compete for the same or a similar enzyme system resulting in a reduced rate of metabolism of thiopental. It is well recognized that such competition for an enzyme system is a common finding for compounds with similar metabolic pathways. An attempt was made during this study to obtain thiopental blood levels in order to clarify the rate of metabolism of thiopental in the presence of alcohol, but the available methods did not give accurate results.

In contrast to the above possible mechanism of action, alcohol in the dose used will result in some central nervous system depression, although this dose in the present experiments did not cause loss of the righting reflex in dogs. In all animals the sleeping time induced by combined alcohol plus thiopental was greater than the sum of the sleeping times induced by either compound administered alone. This indicates a possible synergistic action of alcohol and thiopental administered together.

Summary and conclusions. Thiopental in a subanesthetic dose caused a significant decrease in the reaction time of mice. Alcohol in doses even very near the hypnotic level did not significantly change the reaction time. When the two drugs were given simultaneously, the reaction time was still not significantly changed. The sleeping times of mice, dogs and rabbits given both thiopental and alcohol were significantly longer than the sleeping times when either of the two drugs alone were given.

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Elevation of Left Auricular Pressure in Relation to Ammonium Pulmonary Edema in the Cat.* (19234)

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The acute pulmonary edema that follows the administration of large doses of ammonium chloride in the guinea pig, rat, and less frequently, in the cat has been the subject of investigations by Koenig and Koenig(1-3). These authors did not believe that any final understanding of the mechanisms involved was possible on the basis of their data but felt that impulses traveling over pulmonary sympathetic nerve fibers were of paramount importance.

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It seemed that additional hemodynamic information would be helpful especially as regards pressure phenomena in the left auricle. Early exploratory experiments in the guinea pig and rat revealed a substantial rise in end-diastolic filling pressure of the left ventricle, but these experiments were in general not technically satisfactory and will not be reported here. Despite the fact that the incidence of ammonium pulmonary edema was lower in the cat than in the other two species, our experiments were performed with that animal largely because of the greater ease

with which adequate hemodynamic observations could be made.

Method. Cats of both sexes weighing from 1.7 to 4.0 kg were used. Sodium pentobarbital, 35 mg per kg, was given intraperitoneally and a left thoracotomy incision performed while respiration was maintained by means of a modified Starling pump. After the pericardium was incised and a No. 18 thin-walled needle passed into the left auricle, vinylite tubing was passed through the needle, and the latter was withdrawn. The tubing was secured in place, and the chest was closed with evacuation of air from the chest. A second vinylite tubing was passed 3 to 4 inches up the femoral vein in order to record inferior vena caval pressures. A third vinylite tubing was used for the recording of femoral arterial pressures.† All pressures were recorded with electromanometers(4) which fed into a multichannel recorder that registered in rectilinear coordinates. The zero reference base line was the cat's left auricle. In cats No. 3, 5, 6, 8, 10, 12, and 15 (Table I), an attempt was made to reconstitute the animals to their presurgical status by administering intravenously 10 cc of polyvinyl pyrrolidone between 10 and 20 minutes prior to the injection of ammonium chloride. Fourteen cc per kg of 6% ammonium chloride solution was then injected intraperitoneally. Not infrequently, an additional injection of ammonium chloride was required, and these were given as half the original dose. Immediately after the death of the animal the chest was opened, and the heart and lungs quickly removed for examination. Generalized pulmonary edema was diagnosed when foamy fluid was observed to exude readily from the cut section of each lobe when compressed. Microscopic sections were obtained from the lungs of one of the two cats thought to have gross evidence of generalized pulmonary edema.

Results. The cats died in from 8 to 58 minutes after the initial injection of ammonium chloride. Two cats required one additional injection, and one cat required two additional injections before death occurred.

† Supplied through the generosity of Becton-Dickinson Co., Rutherford, N. J.

TABLE I. Changes in Left Auricular and Vena Caval Pressures After the Intraperitoneal Injection of Ammonium Chloride in the Cat.

	Left auricle		Vena cava	
Cat No.	Control	After NH_4CL	Control	After NH_4CL
1	5	15	2	15
2†	3	11	—	—
3	7	18	—	—
5†	2	10	11	15
6*	7	24	7	15
7	3	20	1	8
8	4	13	2	13
10	5	20	9	19
11	3	18	8	18
12	5	13	4	11
15*	9	40	4	17
16	0	16	3	15
Mean	4.4	18.2	5.1	14.6

* Generalized pulmonary edema.
† See text.

All showed the neuromuscular abnormalities (convulsions and twitching) as previously described(2,3). The control levels listed in Table I were obtained from the immediate pre-injection portion of the tracings. The levels listed in Table I as "after NH_4CL" were obtained by estimating the mean pressure for the period after a significant elevation had occurred and was sustained. This was usually the period of 7 to 12 minutes before death. The immediate (2 to 3 minutes) pre-mortem decline was not included in this estimate.

Arterial pressure either rose or fell immediately after the injection. In all but one cat (No. 15), when arterial pressure rose initially, it fell to or below control levels during the period of elevated left auricular pressure.

After the injection of ammonium chloride, left auricular pressure rose an average of 13.8 cm of water and vena cava pressures 9.5 (Table I). Generalized pulmonary edema was observed in 2 of the 12 cats, No. 6 and No. 15. These 2 cats had had sustained left auricular pressures of 24 and 40 cm of water, respectively. The other 10 cats had sustained left auricular pressures of 20 cm of water or below and did not show generalized pulmonary edema. Two of these 10 had localized edema as follows. Cat No. 2, having been on its right side, had the right lower lobe as the most dependent portion of the pulmonary vascular bed. Although its general sustained

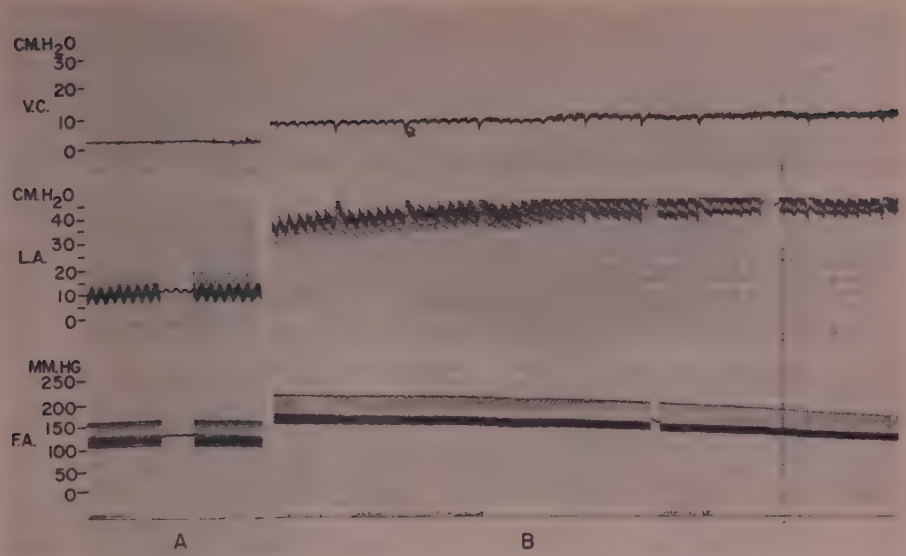

FIG. 1. Cat No. 15. V.C. = vena cava. L.A. = left auricle. F.A. = femoral artery. Chart speed = 1 mm per sec. Solid black lines are electrically integrated (mean) pressures. Others are full pulse pressures. A. One min prior to intraperitoneal injection of ammonium chloride. B. Begins 8 min after injection and ends 3 min prior to death. Generalized pulmonary edema.

left auricular pressure was 11 cm of water, there were several peaks at 27 cm of water during convulsions which lasted between 30 and 60 seconds. At post-mortem a small amount of foam could be expressed from the tip of its right lower lobe. In Cat No. 5 the left middle lobe was traumatized during the operative procedure and was noted to be atelectatic when the chest was closed. Its general elevation of left auricular pressure was 10 cm of water. At post-mortem equivocal edema was present in its left middle lobe. The lungs of all other cats were free of grossly observable edema.

The pre- and post-injection levels of vena cava, left auricle, and femoral arterial pressure from Cat No. 15, which showed generalized pulmonary edema, are shown in Fig. 1.

Discussion. Of the 6 cats examined by Koenig and Koenig(3), 3 developed acute pulmonary edema, while in the above series of 12 cats only 2 developed it. The experimental conditions were different in our group both as regards the administration of a barbiturate adequate for surgical anesthesia and the amount of surgery required. Thus any comparison of the incidence of pulmonary edema is irrelevant. What is thought to be of considerable interest, however, is the fact that in all experiments a significant rise in left auricular pressure did occur after the administration of ammonium chloride and, further, that the 2 cats which developed the highest sustained left auricular pressures (24 and 40 cm of water) were the only ones which developed generalized pulmonary edema.

Two of the several mechanisms considered by Koenig and Koenig, namely, pulmonary venular spasm and a change in pulmonary capillary permeability, are not supported by the above data. Contrariwise, "the mechanical theory of left ventricular failure [was] mentioned only to reject it"(3). It is presumed for the purposes of this discussion that left ventricular failure is a state wherein the left ventricle does not eject blood in such a way as to maintain pulmonary capillary and venous pressures within normal limits. It was felt(3) that left ventricular failure was not important because one rat which developed pulmonary edema did not exhibit arterial hypertension. However, it is a common clinical experience to observe patients in pulmonary edema following a coronary occlusion in

whom the arterial pressure is at or below normal levels.

The fact that dibenamine, a sympatholytic agent, prevented the formation of pulmonary edema(3) is not necessarily an indication that it is blockade of the pulmonary sympathetics which alters the outcome. Cameron and De (5,6) produced rapid, lethal pulmonary edema in the rabbit with the intracisternal injection of thrombin and fibrinogen, and Cruchaud and Vermeil(7) demonstrated that this type of pulmonary edema is also prevented by dibenamine. However, in recent studies on the dog it became clear that the striking elevation of pulmonary capillary pressure that occurs soon after the intracisternal injection of thrombin and fibrinogen is due not to activity of pulmonary sympathetics but rather to impulses traveling over sympathetics to the peripheral vascular bed(8-10). The increased peripheral vascular resistance, as well as the shift of blood from periphery to lung, was shown to be mainly responsible for the elevations of pulmonary capillary pressure that occurred.

Summary. (1) Ammonium chloride was administered intraperitoneally to 12 cats under sodium pentobarbital anesthesia with measurement of left auricular, femoral arterial, and vena caval pressures. (2) Left auricular and vena caval pressures rose significantly in all experiments. Arterial pressures were generally, but not always, lower during the period of the sustained elevation of left auricular pressure than during the control period. (3) Two of the 12 cats developed generalized pulmonary edema. These 2 had sustained left auricle pressures of 24 and 40 cm of water. (4) Although the above experiments do not preclude the possibility that other mechanisms may also be operating, they do suggest that left ventricular failure plays a role in the development of ammonium pulmonary edema in the cat.

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Function and Metabolic Significance of Penicillin and Bacitracin in the Chick.* (19235)

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Elam, Gee, and Couch(1) reported that the fecal microflora of the growing chick was influenced by the feeding of penicillin. These workers reported further that this antibiotic stimulated growth and egg production. Aureomycin has also been shown to increase egg production and hatchability(2). The growth promoting effect of penicillin and terramycin has been explained as an inhibition of *Clostridium perfringens* in the intestinal tract of turkeys by Sieburth *et al.*(3). Halick and

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TABLE I. Supplementation to Basal Diet.

Group	Penicillin					Bacitracin		Vit. B_{12}, μg
	Oral, mg	Inj., mg	Autoclaved, inj., mg	Autoclaved, oral, mg	In sesame oil, units	Oral, mg	Inj., mg	
1	0							
2								1‖
3	33*							
4		1.2†						
5			1.2‡					
6				33				
7					15000§			
8	33							1
9		1.2						1
10			1.2					1
11				33				1
12					15000			1
13						33		
14							1.2	
15						33		1
16							1.2	1

* Per kg of diet. † Inj. per bird per wk. ‡ Autoclaved 15 min at 15 lb. § Inj. per bird every other day. ‖ Inj. per bird per wk.

Couch(4) fed aureomycin and penicillin to hens receiving a purified diet containing soybean protein and sucrose and found that these antibiotics apparently assisted in the depletion of the birds of vit. B_{12} and possibly of an unidentified factor necessary for normal hatchability and embryonic development. Shortly after the isolation of vit. B_{12}(5), the pure vitamin was reported to promote a growth response in chicks equivalent to that obtained with crude sources of the animal protein factor(6,7). It has been demonstrated that unidentified factors over and above vit. B_{12} are necessary for maximum chick growth(8-13). Further, Stokstad and Jukes(14) and Whitehill *et al.*(15) reported that aureomycin, streptomycin, penicillin, and sulfasuxidine promoted the growth of chicks adequately supplied with vit. B_{12}.

This experiment was designed to determine the effect of oral and parenteral administration of penicillin, bacitracin, and autoclaved penicillin on growth and aerobic fecal microflora.

Experimental. The New Hampshire chicks used in these studies were obtained from hens which had been fed a diet composed of 20% ground yellow corn, 45.6% ground milo, 20.5% soybean oil meal, 4% fish meal, 2% oyster shell, 3% alfalfa leaf meal, 2% dried whey, 0.25% Lederle APF-B_{12} concentrate, 0.5% NaCl, and 0.175% fortified fish oil (2250A-300D). In addition 2 mg riboflavin, 5 mg calcium pantothenate, 500 AOAC chick units of vit. D_3, and 100 mg $MnSO_4$ were fed per pound of feed. Four hundred and eighty chicks were randomized into 16 groups of 30 chicks each and were kept in batteries with raised screen floors. Feed and water were supplied *ad libitum*. The birds were weighed at weekly intervals during an experimental period of 10 weeks. The basal diet (G-1) used in this study was the same as that reported earlier by Couch and Olcese(16). The basal diet was fed alone and supplemented as shown in Table I. The autoclaved penicillin used in this experiment was prepared according to information from Dr. David Hendlin(17). Sixty mg of procaine penicillin G was added to 20 ml of water and autoclaved for 15 minutes at 15 lb of pressure at 120° to 125°C. In one assay (disc plate technic) no growth inhibition was observed even when the autoclaved material was tested at undiluted strength.† In the other instance (cup plate procedure) the solution of autoclaved procaine penicillin G showed a potency of less than 0.2 unit per ml (less than 0.07 unit per mg)‡. The procaine penicillin G sample (from original aliquot and used as a standard) assayed 1130 units per mg by microbiological

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‡ Dr. David Hendlin, Merck and Co., Rahway, N. J.

FIG. 1. Effect of oral and parenteral administration of penicillin, autoclaved penicillin, and injecting vit. B_{12} on growth of New Hampshire chicks.

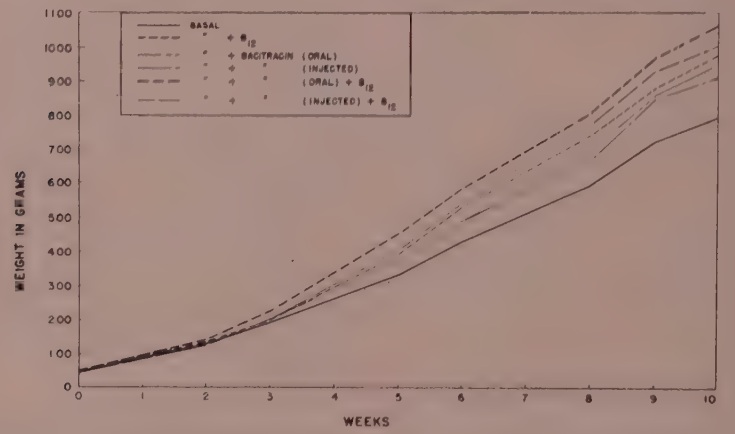

FIG. 2. Effect of oral and parenteral administration of bacitracin and injecting vit. B_{12} on growth of New Hampshire chicks.

assay and 1017 units per mg by chemical assay which are in good agreement with the theoretical value of 1009 units per mg.

Results and discussion. The administration of oral or injected penicillin increased the rate of chick growth (Fig. 1). A somewhat greater growth increase was also apparent when penicillin in sesame oil (containing aluminum monostearate) and autoclaved penicillin were each injected. This is possibly traceable to the fact that penicillin in sesame oil remained in the tissues for a longer period of time and was made available more slowly. The fact that the injection of autoclaved penicillin increased growth is of particular significance since the feeding of autoclaved penicillin had no effect on the growth of the birds. The injection of vit. B_{12} resulted in an increase in growth. When the birds received both vit. B_{12} and penicillin the weight of the birds was increased above that of those which were given B_{12} without penicillin.

Bacitracin administered orally or parenterally produced an increase in growth over that of the birds fed the basal diet unsupplemented (Fig. 2). This increase in growth was about the same as that observed when vit. B_{12} alone was added to the basal diet. Birds which received bacitracin orally or parenterally with vit. B_{12} weighed more at the end of the 10

TABLE II. Summary of Statistical Analysis of Enumerative Data from Fecal Droppings of Chickens from 2-10 Weeks of Age.

		Penicillin						
	Basal	Oral	Inj.	Auto. inj.	Auto. oral	Al mono-stearate	F-value†	LSD§
Penicillin‖	4.91*	6.66	5.75	6.09	6.26	6.67	3.88‡	1.22
Aureomycin¶	2.33	4.06	2.62	3.21	2.72	3.03	3.65‡	.85
Potato-dextrose (yeast)	2.08	3.24	2.24	2.31	2.04	2.36	5.57‡	.59
		Penicillin plus B_{12}						
Penicillin‖	6.03	7.60	5.64	6.18	5.75	7.61	3.88‡	1.22
Aureomycin¶	2.43	3.84	3.31	3.21	3.03	3.25	3.65‡	.85
Potato-dextrose (yeast)	2.27	3.42	2.32	2.32	2.44	2.98	5.57‡	.59

* Avg No. of microorganisms in logarithms. † F-value calculated for all 12 groups. ‡ Significant at the 1% level. § Least significant difference—calculated for all 12 groups. ‖ Penicillin resistant. ¶ Aureomycin resistant.

week test period than did those of any other group.

Effect on flora. The oral administration of penicillin produced an increase in the penicillin resistant organisms, aureomycin resistant organisms, and in the yeast counts (Table II). There was no significant increase in the total dilution count, enterococci, lactics, or coliforms when penicillin was administered orally to the birds over those receiving the basal diet. In an earlier study from this laboratory the oral administration of penicillin increased the total number of bacteria and the enterococci(1). This was not observed in the present study. It should be pointed out that vit. B_{12} depleted chicks were used in the first study(1), whereas chicks from hens on an adequate diet were used in this investigation.

The parenteral administration of penicillin in water did not have any effect on the aerobic fecal microflora. When penicillin was administered in sesame oil, containing aluminum monostearate, there was an increase in the penicillin resistant organisms and in the yeast counts. These data might be explained by the fact that the penicillin in sesame oil was injected every other day and was released slowly. Such a procedure might have led to the excretion of the antibiotic into the intestinal tract and thus an effect on the microflora was produced. The administration of autoclaved penicillin had little effect upon the fecal microflora.

Since the parenteral administration of penicillin and of autoclaved penicillin failed to have any appreciable effect upon the microflora, and yet stimulated the rate of growth, it might be surmised that a fragment of the penicillin molecule may have acted as a metabolite within the body of the bird.

Bacitracin when administered orally or parenterally, with or without vit. B_{12}, failed to have any effect upon the thioglycollate count, enterococci, lactics, yeasts, coliforms, penicillin resistant, or bacitracin resistant organisms. This observation appears to be rather significant in that bacitracin produced a significant increase in growth. Such an observation lends support to the theory that the antibiotic molecule or fragment of same might possibly have stimulated growth by acting as a metabolite within the body of the bird.

Summary. (1) The feeding of penicillin, but not of autoclaved penicillin, stimulated the growth of birds fed an all-vegetable protein diet. The injection of either penicillin or autoclaved penicillin similarly increased growth. (2) Bacitracin when administered orally or parenterally increased the rate of growth. (3) Under the conditions of the experiment reported herein the feeding of penicillin increased the penicillin and aureomycin resistant organisms and increased the yeast count. The parenteral administration of penicillin had no effect on the fecal microflora. Autoclaved penicillin had little effect upon the fecal microflora. Bacitracin when administered orally or parenterally failed to have any effect on the fecal microflora. (4) Since the parenteral administration of antibiotics and autoclaved penicillin increased the rate of growth and yet had little effect on the fecal

aerobic microflora count it seems possible that the antibiotics may have stimulated growth by some other mechanism. Thus, it might be surmised that the antibiotic molecule or a fragment of same might act as a metabolite within the body of the bird.

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Beneficial Effects of Liver on Cortisone Acetate Toxicity in the Rat.* (19236)

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Considerable data are available indicating that, in addition to the known nutrients, substances are present in our diet which may be required in increased amounts during conditions of stress. Such factors are apparently dispensable under normal conditions, or their requirements are so small they may readily be met by amounts present in the diet or through the synthetic activity of the intestinal flora or the animals' own tissues. Certain drugs or other "stress factors" may, however, increase requirements for these substances to such an extent that deficiencies occur, manifested by retarded growth or tissue pathology, and preventable by the administration in appropriate amounts of the missing nutrient (1,2). Whole liver is a potent source of such unknown nutrients. Thus whole liver or fractions thereof has been shown to counteract the deleterious effects of massive doses of strychnine(3), sulfanilamide(4), promin(5,6), atabrine(7,8) and dinitrophenol(9). Similar results have been observed following the administration of toxic doses of diethyl-stilbestrol(4), alpha-estradiol(10), desiccated thyroid(11-14), and thyroxin, thyroglobulin and iodinated casein(15). In the present communication data are presented on the comparative effects of whole liver and the known B vitamins on cortisone acetate toxicity in the immature rat.

Procedure. Exp. 1. The basal ration employed in the present experiment consisted of sucrose, 61%, casein,† 24%; salt mixture,‡

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5%; and cottonseed oil (Wesson), 10%. To each kg of the above were added the following synthetic vitamins: thiamine hydrochloride, 20 mg; riboflavin, 20 mg; pyridoxine hydrochloride, 20 mg; calcium pantothenate, 60 mg; nicotinic acid, 60 mg; ascorbic acid, 200 mg; 2-methyl-naphthoquinone, 10 mg; and choline chloride, 2 g. To each kg of diet were also added 4000 U.S.P. units of vitamin A§ and 400 U.S.P. units of vitamin D.‖ Each rat also received once weekly a supplement of 4.5 mg of alpha-tocopherol acetate. Thirty male and 30 female rats of the Long-Evans strain were selected at 23 to 25 days of age and an average weight of 46.8 g for the following experiment. Animals were placed in metal cages with raised screen bottoms to prevent access to feces and were fed the following diets *ad lib.* (6 males and 6 females per group): (a) basal ration alone; (b) basal ration plus 200 mg cortisone acetate¶ per kg of diet; (c) basal ration plus 400 mg cortisone acetate per kg of diet; (d) basal ration plus 10% whole liver powder** plus 200 mg cortisone acetate per kg of diet; and (e) basal ration plus 10% whole liver powder plus 400 mg cortisone acetate per kg of diet. The cortisone acetate and whole liver powder were incorporated in the basal ration in place of an equal amount of sucrose. Diets were made up weekly and stored under refrigeration when not in use. Animals were fed on alternate days. All food not consumed 48 hours after feeding was discarded. These measures were employed to minimize oxidative changes in the diet. Feeding was continued for 6 weeks or until death, whichever occurred sooner.

Exp. No. 2. The following 3 diets were employed in this investigation: (a) basal ration; (b) basal ration plus the following additional B vitamins per kg of diet: thiamine hydrochloride, 20 mg; riboflavin, 20 mg; pyridoxine hydrochloride, 20 mg; calcium pantothenate, 60 mg; nicotinic acid, 60 mg; biotin, 5 mg; folic acid, 10 mg; p-aminobenzoic acid, 400 mg; inositol, 800 mg; and vitamin B_{12}, 150 μg; and (c) basal ration plus 10% whole liver powder. Cortisone acetate was incorporated in the above diets at a level of 100 mg per kg of ration. The whole liver powder and the various supplements were added in place of an equal amount of sucrose. Experiments were conducted with rats fed the above diets and with animals fed the basal ration with cortisone acetate omitted. Twenty-four male and 24 female rats of the Long-Evans strain were selected at 21 to 24 days of age and were fed the above diets *ad lib.* for a period of 6 weeks (6 rats of each sex per group). The experimental procedure was similar to that previously described.

Results. Exp. No. 1. Findings on growth and survival are summarized in Table I. Gain in body weight was significantly reduced in all rats fed cortisone acetate-containing diets. The retardation in growth was particularly marked for female rats and was proportional in general to the level of drug fed. The weight increment of cortisone acetate-fed rats was significantly greater for rats fed the liver-containing diet than for animals fed the basal ration. This occurred at both levels of drug employed and in both male and female rats. The growth-promoting effect of liver was relatively greater in the female than in the male. A beneficial effect of liver was similarly noted in respect to survival. During the 5th and 6th week of feeding a number of deaths occurred in cortisone acetate-fed rats on the basal ration. The mortality incidence was particularly marked at the higher dosage level and was greater in females than in males. No deaths occurred in any of the rats fed cortisone acetate in conjunction with the liver-containing ration.

Alopecia occurred in all cortisone acetate-fed rats on the basal ration, particularly in females. By the end of the first week of feeding, loss of hair was marked between the ears and on the dorsal surface of the neck

† Vitamin Test Casein, General Biochemicals, Chagrin Falls, O.

‡ Hubbel, Mendel and Wakeman Salt Mixture, General Biochemicals, Chagrin Falls, O.

§ MYVA-DRY Powder, Distillation Products, Rochester, N. Y.

‖ HY-DEE Powder, Standard Brands, New York.

¶ The cortisone acetate was kindly provided by Dr. R. A. Peterman of Merck and Co., Rahway, N. J.

** Desiccated Liver, Armour & Co., Chicago, Ill.

TABLE 1. Beneficial Effects of Liver on Growth and Survival of Immature Rats Fed Toxic Doses of Cortisone Acetate (6 Animals per Group).

Dietary group	Initial body wt, g	Gain in body wt after following days of feeding, g 14th	28th	42nd	% surviving
Exp. 1	Males				
BR*	46.3	56.6 (6)	149.7 (6)	160.7 (6)	100
BR + 200 mg CA† per kg of diet	50.3	4 (6)	33 (6)	44.1 (6)	100
BR + 200 mg CA per kg diet + 10% whole liver	47.7	13.7 (6)	48.4 (6)	81.6 (6)	100
BR + 400 mg CA per kg of diet	51.2	—3.1 (6)	3.7 (6)	8 (2)	33.3
BR + 400 mg CA per kg of diet + 10% whole liver	48.3	1.7 (6)	22.8 (6)	43.5 (6)	100
Exp. 2					
BR	41.6	54.8 (6)	115.7 (6)	152.3 (6)	100
BR + 100 mg CA per kg of diet	42.4	30.8 (6)	70.3 (6)	108.3 (6)	100
BR + 100 mg CA per kg of diet + B vit.	42.3	31.8 (6)	72.8 (6)	108.8 (6)	100
BR + 100 mg CA per kg of diet + 10% whole liver	41.8	53.3 (6)	114.8 (6)	161.8 (6)	100
Exp. 1	Females				
BR	43.4	46.5 (6)	83.7 (6)	115.7 (6)	100
BR + 200 mg CA per kg of diet	45.4	—1.8 (6)	7.1 (6)	4 (4)	66.7
BR + 200 mg CA per kg of diet + 10% whole liver	44.5	6.3 (6)	32.4 (6)	55.5 (6)	100
BR + 400 mg CA per kg of diet	46.3	—6.1 (6)	—7 (6)	—14 (1)	16.7
BR + 400 mg CA per kg of diet + 10% whole liver	44.8	.2 (6)	18.2 (6)	29.5 (6)	100
Exp. 2					
BR	40.5	42 (6)	70.5 (6)	96 (6)	100
BR + 100 mg CA per kg of diet	41.7	11 (6)	43.5 (6)	64.5 (6)	100
BR + 100 mg CA per kg of diet + B vit.	41.2	14.5 (6)	47.5 (6)	80 (6)	100
BR + 100 mg CA per kg of diet + 10% whole liver	41	33 (6)	71 (6)	98 (6)	100

‡ Values in parentheses indicate the No. of animals which survived and on which averages are based.

* BR = Basal ration. † CA = Cortisone acetate.

of all the above rats. After 28 days of feeding 4 out of 6 female rats and 1 out of 6 male rats fed cortisone acetate at a level of 400 mg per kg of diet were completely denuded with the exception of several strands of fur along the paws (Fig. 1). Marked alopecia although less extensive than the above also occurred in rats fed this drug at a level of 200 mg per kg of diet and in the remaining animals fed this drug at the higher level. Alopecia was reduced both in incidence and degree in all cortisone acetate-fed rats on the liver-containing ration, particularly in males. Priapism was noted after the 4th week of feeding in a number of cortisone acetate-fed rats both on the basal and liver-containing ration.

During the 6th week of feeding total and differential white cell counts, hemoglobin determinations, total red cell counts and direct eosinophil counts were made on the tail blood of all surviving rats. Differential counts were made on smears stained with Wright's stain, 100 cells on each of 2 slides being employed for each analysis. No significant difference in erythrocyte count or hemoglobin level was observed between the various groups, erythrocytes averaging 7.5 to 8.6 million per cc and hemoglobin 17.1 to 18.4 per 100 cc. A significant reduction in eosinophiles and lymphocytes and an increase in polymorphonuclear leukocytes occurred in all cortisone acetate-fed rats both on the basal and liver-containing ration. Eosinophiles dropped from an average of 350 cells per cc for rats fed the basal ration to less than 50 cells per cc for animals fed cortisone acetate-containing diets; while lymphocytes fell from an average of 7800 per cc to less than 5000 per cc (average range 2100 to 5000 per cc). The reduction in lymphocyte count was greater in females than in males and was proportional in gen-

FIG. 1. Litter mate male rats after 40 days of cortisone acetate administration. The rat in the foreground was fed a purified ration containing 400 mg cortisone acetate per kg of diet. The rat in the background was fed a similar diet supplemented with 10% whole liver powder.

eral to the level of drug fed. Polymorphonuclear cells increased from an average of 2700 per cc for rats on the basal ration to averages exceeding 5200 per cc for animals fed cortisone acetate. Changes in total WBC as a result of cortisone acetate administration were not statistically significant. No significant differences in respect to the above were observed between animals fed the basal ration and those fed a similar diet supplemented with whole liver powder.

Exp. No. 2. Findings are summarized in Table I. Cortisone acetate at a level of 100 mg per kg of diet significantly reduced the gain in body weight of immature male and female rats fed a purified basal ration. Supplements of the known B vitamins were without significant effect. Desiccated whole liver at a level of 10% of the diet completely counteracted the above retardation in growth. Neither alopecia nor priapism occurred in any of the rats fed this level of cortisone acetate.

The mean arterial pressure was determined by the direct method in 3 male and 3 female rats in each group on the 43rd day of feeding.†† The rats were anesthetized with ether and the abdominal aorta was exposed and punctured with a 24-gauge needle connected to a mercury manometer of 1 mm bore tubing. The puncture was made during light anesthesia and with the mercury column in the manometer raised to a level of 90-100 mm Hg. No significant difference in mean arterial pressure was observed between the various groups (values averaged between 72 and 76 mm Hg for the different groups with an individual range between 68 and 81 mm Hg).

Discussion. The toxic effects of large doses of cortisone acetate were largely counteracted in the rat by the administration of desiccated whole liver. Liver, however, did not counteract the effect of the drug on the leukocyte count. The beneficial effect of liver was apparently not due to its content of known B vitamins. This is indicated by the fact that supplements of all the known B vitamins were without significant effect on the gain in body weight of immature rats fed 100 mg per kg of diet of cortisone acetate. Available data do not indicate, however, whether the protective effect of liver was due to an unidentified factor(s) or to its content of other known nutrients. Since liver also contains a factor (distinct from any of the known B vitamins) which counteracted the toxic effects of thyroid(11-14), promin(5,6) and atabrine(7) administration in the rat, further work is indicated to determine what relationship if any exists between the "antitoxic factor of liver"(2) and the factor or factors indicated above.

Summary. The administration of toxic

†† We are indebted to Dr. Sheldon Rosenfeld, Dept. of Physiology, University of Southern California, for the blood pressure determinations.

doses of cortisone acetate to immature rats maintained on a synthetic diet resulted in retarded growth, alopecia and death. These effects were largely counteracted by the administration of desiccated whole liver when fed at a level of 10% of the diet. The protective factor(s) in liver was distinct from any of the known B vitamins.

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Further Studies on Anti-Thyroxine Effects of Various Compounds.* (19237)

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The extension by Woolley(1) of the specific metabolic inhibitor principle into thyroxine analogues initiated an interest which has been taken up by other laboratories, reports of whose work have appeared during the past few years. Frieden and Winzler(2) expanded the variety of substituted ethers of 3,5-diiodo-4-hydroxybenzoic acid, demonstrating special activity in 4-benzyloxy-3,5-diiodobenzoic acid and 4-benzyloxy-3,5-diiodophenylalanine in preventing the thyroxine-induced acceleration of tadpole metamorphosis. Maclagan, Sheahan and Wilkinson(3) noted interference with increased metabolic rates caused by thyroxine in mice when 4-benzyloxy-3,5-diiodobenzoic acid was injected, as well as the dimethyl acetal of 3,5-diiodoanisaldehyde. As was also pointed out by Barker *et al.*(4), the amounts of inhibitors required for mammalian experiments were greater than for amphibia. Cortell(5) was able to cause a considerable reversal of the thyroxine depression of thiouracil-induced hypertrophy of rat thyroid glands by the use of 2′,6′-diiodothyronine. Barker and coworkers(4) presented the results of 3 different approaches to quantitative evaluation of thyroxine inhibition, two involving measurement of changes in metabolism and the third employing the depression of response to standard estrogen dosages produced by thyroxine. It was concluded that the thyroidectomized rat maintained at an approximately normal metabolic level by daily injections of 12 μg of DL-thyroxine† offered a highly sensitive preparation for evaluation of antithyroxine effects. This technic was used by Klitgaard and coworkers(6) in their study of iodinated phenoxyacetic acids, several of which were found to antagonize the energy metabolism control of the thyroid hormone, with greatest activity when iodine was in the 2, 4, and 6 positions in the phenyl ring.

The present investigation was concerned with a varied group of substances which either have been reported to show thyroxine inhibition or might be expected to do so.

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† DL-Thyroxine was made available by Dr. K. W. Thompson, Organon, Inc., Orange, N. J.

Experimental methods. The general procedure was that described by Klitgaard *et al.* (6), which should be consulted for details. The effectiveness of thyroxine antagonism was calculated as the percentage of return of the thyroxine-maintained metabolic rate of thyroidectomized rats produced by a daily dose of the compound being studied. These values were obtained by measuring the oxygen consumption 4 weeks or more after thyroidectomy and after the athyroid animals had been maintained on 12 μg of thyroxine per kg body weight per day. Metabolic rates were then determined one week and 2 weeks after commencing administration of the material, and the 2 values averaged unless there was a continuing downward trend, in which case the 2-week value was used, or the experiment continued another week. In order to be as certain as possible of the genuineness of the change, the animal's metabolic rates usually were determined for several weeks after cessation of drug administration, until plateauing had occurred. This value was averaged with the preliminary figure. With the exception of iodothiouracil, which was fed after thorough mixing with dry powdered diet, the substances being tested‡ were injected subcutaneously, in saline, propylene glycol (PG) or sesame oil solution, using 0.5 to 1.0 cc per kg body weight per day. Dosages of specific compounds are shown as number of times isomolar with the dose of 12 μg of thyroxine injected per kg per day to maintain normal metabolic rates in thyroidectomized rats.

Results and discussion. The results obtained are shown in Table I. Each set of data represent 4 animals, since the metabolism apparatus used was set up for this number. Control groups of rats, injected with saline, PG or sesame oil, showed variations from their basal oxygen consumption values of about ±10%. When these data were recalculated as though some potential inhibitor compound were being evaluated, "antagonisms" of —3 to —24% (*i.e.*, "thyroxine-like" action) and +6 to +21% were obtained, indicating that differences of less than 20% can probably be ignored as to be anticipated from spontaneous fluctuations.

Vit. A (synthetic crystalline material administered as the palmitate) revealed unmistakable effects at 300 times isomolar, equivalent to 4416 I.U. of vit. A per kg per day. The depression was somewhat higher at 500x, and considerably so at 1000x, indicating a competitive type of inhibition. The effects were always more marked after 2 weeks of administration than after one, and the metabolic rates invariably remained depressed for at least one week after injections were stopped, a phenomenon probably resulting from hepatic storage of the vitamin. β-Carotene was less effective than the oil solution of vit. A, suggesting that conversion to the biologically active form was required. The basis for these effects must lie outside of the ordinary functions of the vitamin, since the animals were fed a basal diet rich in vit. A; furthermore, the 100x dosage exerted no thyroxine inhibition, even though it furnished 1472 I.U. of vit. A per kg of body weight per day above the amount in the diet. Several reports have previously discussed the ameliorating effects of large quantities of vit. A upon clinical as well as experimental hyperthyroidism(7,8). In addition, the inadequate hepatic conversion of carotene in hypothyroidism has been well established(9), but it is very doubtful that these two processes can be related.

Folic acid was found ineffective, and no effects of vit. B_{12} could be demonstrated con-

‡ Thanks are due the following for furnishing compounds for testing: Dr. M. J. Schiffrin, Hoffmann-LaRoche, Inc., for Vit. A palmitate in oil and in saline, ribofuranoside of 5, 6-dimethylbenzimidazole (Ro 2-3950), 5,6-dimethylbenzimidazole, and N- [4-(4′-hydroxyphenoxy)-phenyl]-glycine (Ro-2-3137); Dr. Cecil R. Kemp, Flint, Eaton and Co., for folic acid and Vit. B_{12}; Dr. R. J. Winzler, University of Southern California, for 4-benzyloxy-3, 5-diiodophenylalanine; Dr. N. F. Maclagan, Westminster Medical School, London, for the 3,5-diiodoanisaldehyde dimethyl acetal and butyl-3, 5-diiodo-4- hydroxybenzoate; Dr. Domenick Papa, Schering Corp., for the rest of the series of 3,5-diiodo-4-hydroxyphenyl substituted compounds; Knoll A.-G. Chemische Fabriken, Ludwigshafen am Rein, Germany, for 3-fluoro-4-hydroxyphenylacetic acid ("Capacin"); Dr. S. C. Wang, State University of Iowa, for 2,4,6-triiodophenoxyacetic acid. Commercial sources were used for the remaining substances.

TABLE I. Effect of Various Compounds on Oxygen Consumption of Thyroxine-Maintained Thyroidectomized Rats.

Inhibitor		Oxygen consumption, cc O_2/100 g/hr			
Compound	Dosage*	Pre-T	On T	T + inhib.	Inhib. of T, %
Saline only		71.8	103.7†	96.9	21.3
		69.8	97.4†	103.9	—23.6
Sesame oil only		70.9	101.1†	95.4	18.9
PG only		67.4	99.4†	97.5	6
		70.1	98.3†	99.2	—3.1
Vit. A palmitate in oil solution	100	70.9	100.7†	100	2.3
	100	68.2	90.9	94.6	—16.3
	300	68.2	102.2†	89.1	38.5
	500	70.9	100.7†	92.7	26.8
	500	68.2	102.2†	88.5	40.3
	500	65.8	97.1†	80.4	53.4
	1000	68.2	98.7†	75.8	75.1
Vit. A palmitate in saline emulsion	500	65.8	98.9†	92.5	19.3
	2000	65.8	98.9†	90.1	26.6
β-Carotene	500	68.6	102.1†	102	.3
	1000	68.6	102.1†	86.9	45.4
Folic acid	500	74.6	102	102.7	—2.6
Vit. B_{12}	1*	74.6	83.4	83.8	—4.5
	10	74.6	100.1†	98.2	7.5
	10	74.6	92.4†	86.9	30.9
	10	66.3	96.8†	88.9	25.9
Ribofuranoside of 5,6-dimethylbenzimidazole (R_o 2-3950)	500	69.3	100.1†	95.9	13.6
5,6-Dimethylbenzimidazole	500	66.3	95 †	91.8	11.1
3,5-Diiodotyrosine	500	71.5	91.2	98.5	—37.1
3,5-Dibromotyrosine	500	69.3	96.3†	93.5	10.4
3,5-Diiodo-2-hydroxybenzoic acid	500	74.6	98.9	91.8	29.2
" 4 " "	500	74.6	94.4†	88	32.3
4-Benzyloxy-3,5-diiodo-phenylalanine	100	68	94.3†	89.5	18.3
	500	68	94.3†	95.8	—5.7
3,5-Diiodoanisaldehyde dimethyl acetal	500	70.1	98.6†	100.7	—7.4
	2000	69.8	100.6	93	24.7
Butyl-3,5-diiodo-4-hydroxybenzoate	500	70.7	100.3†	88.7	39.2
	500	73.3	98 †	86.2	47.8
	2000	68.5	103.1†	89.5	39.3
2-(3,5-Diiodo-4-hydroxyphenyl)-benzoic acid	500	65.7	93.4	92.9	1.8
3,5-Diiodo-4-hydroxyphenylacetic acid	500	64.6	97.2	106.5	—28.5
α-Methyl-3,5-diiodo-4-hydroxycinnamic acid	500	70.7	102.3†	93.8	26.9
α-Methyl-β-(3,5-diiodo-4-hydroxyphenyl)-propionic acid	500	70.6	98.6†	81.6	60.7
α-Phenyl-β- " ("Priodax")	500	72.1	96.7	86.9	39.8
	500	66.7	97.5†	93.1	14.3
γ-(3,5-Diiodo-4-hydroxyphenyl)-butyric acid	500	70.7	103 †	92.4	32.8
ω-(3,5- " hexoic "	500	67.8	91.4†	84.1	30.9
ω-(3,5- " caprie "	500	69.5	100 †	88.6	37.4
3-Fluoro-4-hydroxyphenylacetic ("Capacin")	500	67.6	96.1†	85	38.9
	2500	66.2	95.9†	88.6	24.6
5-Iodo-2-thiouracil	500	70.9	99.3†	86.8	44
	500	72.9	100.3†	92.4	28.8
	500	70	102.1†	91.7	32.4
	2000	70	102.1†	91.1	34.3
2,4,6-Triiodophenoxyacetic	100	70.8	107.3†	100.9	17.5
	100	73.3	95.7†	93.5	9.8
	300	70.8	108.6†	96.5	32
	300	73.3	95.7†	87.4	27.1
	500	73.3	101.7†	87.4	50.4
Cholesterol	500	70	103 †	80.2	69.1
N-[4-(4'-Hydroxyphenoxy)-phenyl]-glycine (R_o 2-3137)	500	72.9	98.7†	101.6	—11.2

* Dose expressed as molar ratio of inhibitor to thyroxine maintenance dose; latter was 12 μg/kg/d. in all instances except the one indicated, which was 6 μg/kg/d.

† These values are averages of metabolism values after withdrawal and before administration of inhibitor.

vincingly at the low molar ratios employed (1x to 10x). The larger of these two doses involved the use of 250 μg B_{12} per kg per day; larger quantities were not tried. Several laboratories have noted that the growth rates of young rats are adversely affected by inclusion of large amounts of desiccated thyroid substance in the food mixture, an interference which could be removed by the addition of liver to the diet (Ershoff)(10). At various times, the active substance has been reported as folic acid and vit. B_{12}. Although it now seems probable that some other substance may be concerned (Ershoff)(11), another factor could be the known increase in requirement for many substances needed for growth in hyperthyroid rats (Drill)(12). Since our animals were not in an active growth phase, such a complication should not enter. Two products of fragmentation of vit. B_{12}, the ribofuranoside of 5,6-dimethylbenzimidazole (Ro 2-3950) and 5,6-dimethylbenzimidazole, proved without effect at 500x, although the metabolic rates of the animals receiving the latter compound dropped 44% one week after injections had been stopped and rose only slowly over the next three weeks to the pre-injection level.

Because of its relationship to thyroxine, the 3,5-diiodo-4-hydroxyphenyl substitution has been placed in many compounds, a high proportion of which exhibit thyroxine antagonism. Outstanding in this regard are the previously reported 4-benzyloxy-3,5-diiodobenzoic acid(2-4) and N-(3,5-diiodo-4-hydroxybenzoyl)-3,5-diiodotyrosine(4), as well as α-methyl-β-(3,5-diiodo-4-hydroxyphenyl)-propionic acid, butyl-3,5-diiodo-4-hydroxybenzoate, ω-3,5-diiodo-4-hydroxyphenyl)-capric acid and γ-(3,5-diiodo-4-hydroxyphenyl)-butyric acid, results of which are shown in Table I. In contrast, several other compounds also containing this grouping showed little or no activity, including 3,5-diiodotyrosine and 4-benzyloxy-3,5-diiodophenylalanine, the latter reported highly effective in tadpoles by Frieden and Winzler(2). A marked species difference has previously been noted to exist between the sensitivity of tadpole and rat to both thyroxine-like and thyroxine-inhibiting analogues, the former reacting to much smaller amounts that the latter(4,13). This difference may be due partially to the different criteria used for evaluation, namely, metamorphosis in the tadpole and metabolic rate changes in the rat.

2 - (3,5-diiodo-4-hydroxyphenyl) - benzoic acid exhibited no thyroxine inhibition, nor did 3,5-diiodo-4-hydroxyphenylacetic, although the latter falls structurally between 3,5-diiodo-4-hydroxybenzoic and γ-(3,5-diiodo-4-hydroxyphenyl)-butyric acids, both of which exhibited about 33% inhibition. The dimethylacetal of 3,5-diiodoanisaldehyde was found much less effective than was indicated by the results of Maclagan, Sheahan, and Wilkinson (3), that inhibition of 55% of the thyroxine effect was caused by a molar ration of 900x. The butyl-3,5-diiodo-4-hydroxybenzoate exhibited a much more pronounced antagonism than the diiodoanisaldehyde, but appeared less than would be expected from the 82% decrease reported by Sheahan, Wilkinson, and Maclagan(14) for 460x and 56% for 22x. Its antagonism in the present series was non-competitive. The English workers used a single massive thyroxine dose in normal animals, which we have found a considerably less satisfactory technic than the maintenance of thyroidectomized, wherein changes are followed over a longer period of time.

"Capacin" (3-fluoro-4-hydroxyphenylacetic acid), the subject of a favorable report in the German clinical literature(15), was found to exert some non-competitive inhibition. In view of the toxicity of the fluoride ion, such an effect might be due to splitting of the organic fluoride. Cortell(5) found only thyroxine-like activity from fluoroiodothyronines, with F substituted for one or more of the iodines.

5-iodo-2-thiouracil administered orally, mixed in the diet because of its insolubility in the solvents employed, produced an appreciable anti-thyroxine action, which appeared to be non-competitive. 2,4,6-triiodophenoxyacetic acid was found to exhibit an increasing amount of antagonism to thyroxine as the dose was increased. The 50.4% inhibition obtained at 500x was, however, appreciably less than the marked 80% previously reported for this compound (Klitgaard *et al.*)(6).

Because of the well known alterations of

TABLE II. Summary of Most Active Thyroxine Antagonists as Studied in this Laboratory.

Compound	Dosage*	Inhibition, %
3-Iodophenoxyacetic acid	500	56
2,4-Diiodophenoxyacetic acid	500	46
2,6- " "	500	67
2,4,6-Triiodophenoxyacetic acid	500	66
Butyl-3,5-diiodo-4-hydroxybenzoate	500	44
4-Benzyloxy-3,5-diiodobenzoic acid	1000	66
α-Methyl-β-(3,5-diiodo-4-hydroxyphenyl)-propionic acid	500	61
N-(3,5-diiodo-4-hydroxybenzoyl)-3,5-diiodotyrosine	500	82
β-Carotene	1000	45
Vit. A	1000	75
Cholesterol	500	69

* Doses expressed as molar ratio of inhibitor to thyroxine maintenance dose of 12 μg DL-form/kg/d.

cholesterol metabolism in hypo- and hyperthyroidism, the 69% reversal of thyroxine effect produced in the single series thus far attempted is a striking observation. It amplifies the observation of Marx, Meserve, and Deuel(16) that addition of cholesterol to rats' diets containing desiccated thyroid would protect the animals against the deleterious effects of the large quantities of hormone. Further exploration is obviously required.

As a general summary of the thyroxine antagonism work of this laboratory to date, Table II shows those compounds which have exerted about 50% or greater interference by the metabolic technic of survey discussed in this paper. It can be seen that an inhibitor: thyroxine ratio of about 500x is required to produce such an effect, in contrast to ratios in the neighborhood of 10x and 20x, as reported by others(2,13). No clear response has ever been obtained at less than 300x, the very slight effects at 10x with vit. B_{12} (averaging 21% inhibition) being merely suggestive. It is easy to picture a blocking action of compounds containing the 3,5-diiodo-4-hydroxyphenyl grouping in accord with the general principles of specific metabolic inhibition, but no consistent pattern of response has yet emerged covering active and inactive positions in aliphatic or aromatic compounds. A theory of response to iodinated phenoxyacetic acids has been evolved(6), to some extent coordinating the activity of this structure with that of the diiodohydroxyphenyl radicle discussed above. The presence of vit. A and cholesterol in the list of active compounds indicates a variety of structure which may require a still more flexible concept of inhibitor action than any yet proposed. As recently discussed in a review(17), it is still impossible to attribute to thyroxine a specific function in any enzyme system or series of systems, so the locus of action of any thyroxine antagonist must also remain in doubt for the present.

Summary. (1) The study of compounds inhibiting the peripheral metabolic action of thyroxine in thyroidectomized rats has been extended to such substances as vit. A, vit. B_{12}, cholesterol and a series of aliphatics and aromatics carrying the 3,5-diiodo-4-hydroxyphenyl grouping. (2) The compounds found capable of producing approximately 50% inhibition were vit. A, β-carotene, cholesterol, butyl-3,5-diiodo-4-hydroxybenzoic acid, and α-methyl-β-(3,5-diiodo-4-hydroxyphenyl)-propionic acid. Several other substances were found to be less active than these. No compound tested was found clearly active as a thyroxine antagonist at an inhibitor:thyroxine ratio of less than 300.

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Relation of Sperm Morphology to Genotypically Controlled Variations in Fertility. (19238)

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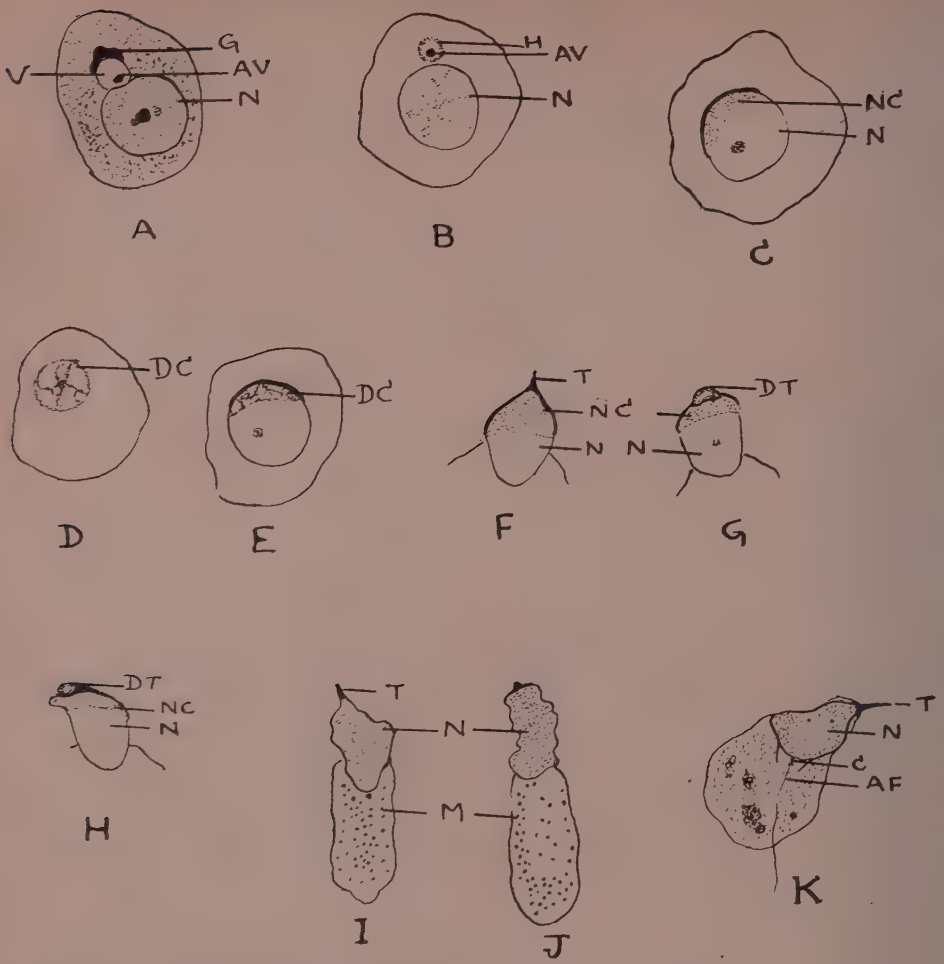
Since the classic investigations of the relation between sperm morphology and impaired fertility made by Williams and Savage(1), Moench(2), and Lagerlof(3), studies of sperm cytology have provoked great interest and some controversy.

In the laboratory mouse a number of mutations have been found, which by their interaction affect the reproductive efficiency of the male in various degrees—from almost complete sterility to almost complete fertility, while their sisters of the same genotype are normal in fertility. The factors responsible for this impairment have been described in several papers from our laboratory(4). Here it need only be recalled that these mutations belong to a series of alleles at or near the T locus in Chromosome IX. Of these alleles 7 have been studied in some detail. They have been normally maintained in balanced lethal lines in which T/t^x is tailless and T/t^n is similarly tailless, whereas crosses between these lines produce compounds t^nt^x distinguished by normal tails and impaired fertility in the males. Examination by Bryson(5) of spermatozoa from males of one of these gene combinations, t^0t^1, which was completely sterile, revealed that, although these sterile males copulated normally and produced a large number of spermatozoa, about a quarter of the sperm had abnormally shaped heads, which were less frequently found in fertile males. He also logically concluded that the normal looking spermatozoa of these sterile males were incapable of fertilization. Recently, spermatozoa have been examined from males of 2 other gene combinations, t^0t^3, and t^1t^3. These males, unlike t^0t^1 males, are not quite sterile. These "quasi-sterile" males produce less than 20% the number of young sired by "normal" males. Attempts to correlate percentages of morphologically abnormal spermatozoa with the breeding efficiency of such males reveal that, while one may with some caution distinguish between normal fertile males and t^0t^3 males whose fertility is impaired in various degrees, any attempt to set numerical boundaries to distinguish absolute sterility from quasi-sterility would be highly misleading.

The average percentage of abnormal sperm in fertile controls is 8.18 ± 0.98, in quasi-steriles 32.20 ± 1.14, and in steriles 43.16 ± 3.69. While the difference in the average percentages of abnormals, between quasi-steriles and steriles, is significant, there is an overlap in their ranges. There is the further evidence that there is no correlation between percentages of abnormal sperms and degree of sterility. All one could safely say is that a higher incidence of abnormal sperms indicates lowered fertility, and there is no justification for treating morphology of spermatozoa as the only diagnostic factor for impaired fertility. The source of the alterations in the sperm pathology was sought by the study of spermatogenesis in quasi-sterile and sterile males. No major cytological aberrations were found.

It has become apparent of late that several different anatomic components of the spermatozoon are significant in evaluating fertility. With improved staining technics, it is becoming clear that nuclear and acrosomal ab-



All figures were drawn with the aid of camera lucida, × 1500. Lettering: AC, acrosome; ACS, space in the acrosome; AF, axial filament; AV, acrosome vesicle; C, centrioles; DC, degenerating cap of the acrosome; DT, degenerating tassel of the acrosome; G, Golgi material; GR, Golgi remnant; H, halo of acrosome; MS, mitochondrial sheath; N, nucleus; NC, normal cap of the acrosome; R, refractive body in the sperm head; SH, sperm head; T, normal tassel of the acrosome; V, vacuole surrounding the developing acrosome. A, K, L, M, N, S from Flemming (without acetic acid), haematoxylin and Fastgreen preparations. B, C, D, E, F, G, H, O, P, Q, R from San Felice and periodic acid Schiff preparations. I, J from Champy-Kull preparations. T, U, V, W, X from basic Fuchsin Fastgreen (Slizinsky's) preparations.

FIG. 1. Comparison of normal and abnormal, early and mid-spermatic stages. A, B, C, normal young spermatids; D, polar view of degenerating cap of a young spermatid; E, side view of same; F, normal sperm head at mid-spermatid stage; G, the same with a degenerated tassel; H, I, J, K, mid-spermatid stages showing abnormalities of nucleus and acrosome.

normalities are more common than abnormalities of the posterior extremity which are comparatively rare(6). After a detailed study of spermiogenesis and mature sperm in quasisteriles it was clear that in the spermatozoa of t^3t^0 males only the nucleus and acrosome were affected. The abnormalities of the nucleus are indicated by the shape and size. (Fig. 2, M, N, U, V, W, X) The axes of the sperm-head are affected. The nucleus assumes a variety of shapes ranging from almost normal to highly distorted. Microsperms, megalo-

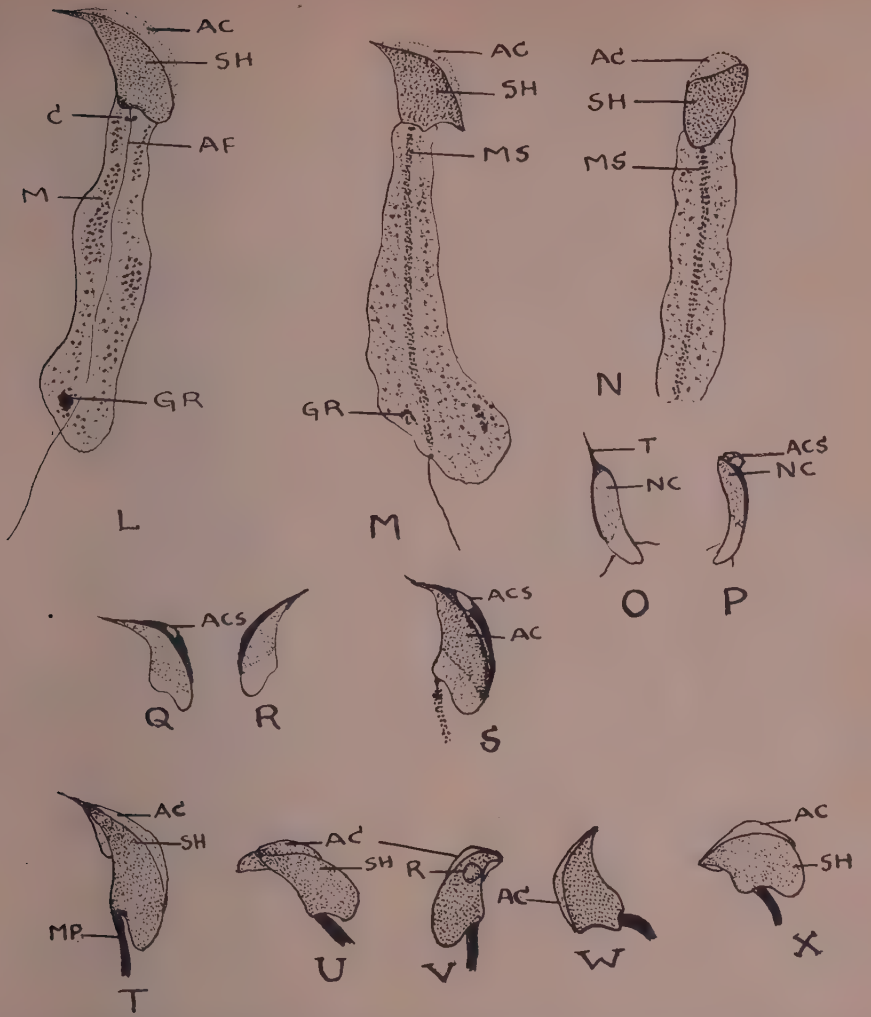


FIG. 2. Comparison of normal and abnormal late spermatid stages and mature sperms. L, a normal late spermatid; M, N, late spermatids showing abnormalities in the shape of the nucleus and acrosome; O, A normal elongating nucleus of late spermatid stage with a normal tassel and cap; P, a normal elongating nucleus with acrosomal spaces; Q, S, normal mature sperm heads with acrosomal spaces (vacuoles); R, T, normal mature sperm heads; U, V, W, X, abnormal sperm heads with abnormal acrosomes.

sperms, and sperms with twin heads are comparatively rare. The nuclear abnormality sets in at a stage when the nucleus of the spermatid has started elongating. (Fig. 1, H, I, J, K).

Studies on the acrosomal changes in spermiogenesis were facilitated by using Periodic-Acid Schiff Reaction(7). Changes in the acrosome consist of the loss of typical sickle shape and of deficiencies in development which lessen in various degrees its visible surface area (Fig. 2, U, V, W, X). In some only a thin fringe of acrosome overlies the flattened anterior nuclear pole (Fig. 2, M, W). The onset of acrosomal abnormality appears to occur in two ways. First, it may set in very early in spermiogenesis, when the acrosome vesicle and the halo around it start spreading

on the anterior pole of the nucleus (Fig. 1, D, E, G); secondly, the abnormality may set in during the midspermatid stage when its topography is disturbed mechanically by the changing nucleus (Fig. 1, H, I, J, K). It was also noted that in some of the apparently normal looking spermatozoa, the acrosome, while it had the typical sickle shape, had one, and rarely two, vacuoles (Fig. 2, P, Q, S) comparable to those of human spermatozoa(8) and those of the bull(9). While these vacuoles were rarely seen, and then with difficulty, using ordinary technics, P.-A.S. technic made it possible to count them in sections of testis.

Conclusions. Most of the sperms in the sterile individuals of these genotypes were normal looking and presumably physiologically inefficient. I agree with Chang(10) in stating that a morphologically normal sperm may be physiologically incapable of effecting fertilization. The fact that the acrosome alterations as revealed by P.-A.S. technic may easily be overlooked when treated by usual methods suggests that differences in such non-chromosomal elements may be of considerable importance in evaluating morphological normality of a spermatozoon.

I am deeply indebted to Prof. L. C. Dunn for his encouragement and help in the preparation of this manuscript.

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Effect of Cortisone on Histamine Liberation Induced by Tween in the Dog.* (19239)

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The inhibitory effect of cortisone on certain experimental hypersensitivity reactions has been shown by several investigators. Anaphylaxis in the mouse(1,2), the Arthus phenomenon in the rabbit(3,4), the tuberculin reaction in the guinea pig(5,6), and the Shwartzman reaction in the rabbit(7) represent hypersensitivity reactions which are influenced by cortisone. In addition, clinical studies too numerous to mention indicate that improvement may follow the administration of cortisone in a variety of human diseases in which hypersensitivity appears to play a part. Somewhat paradoxically, anaphylactic shock cannot be prevented in the guinea pig(8,9) with cortisone administration, although certain features of passive anaphylaxis in this species may be modified *in vitro*(10). Also, passive anaphylaxis in the dog(11) is uninfluenced by

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cortisone treatment. Despite extensive investigations, the mechanism of action of cortisone in hypersensitivity has not been established. From the available evidence, it is not clear whether the important action of cortisone is exerted on the immune mechanisms involved or on the liberation and effect of pharmacologically active compounds which may be released by antigen-antibody reactions.

In view of the lack of understanding of the mode of action of cortisone in hypersensitivity, it seemed worthwhile to study the possible effect of this drug on the reaction induced by Polyoxyethylene derivative of sorbitan monolaureate (Tween 20) in the dog. This remarkable phenomenon, first reported by Krantz and coworkers(12,13), shows many similarities with anaphylaxis in the dog, including the liberation of a histamine-like substance. It is, however, a simpler phenomenon than anaphylaxis, since no antibodies or antigen-antibody reactions are involved. Because of the relative simplicity of the Tween phenomenon, it seemed likely that any effect of cortisone on it would be more subject to analysis in terms of mechanisms involved than the effect of cortisone on anaphylactic hypersensitivity. The data which form the basis of this report indicate that the compound liberated in the dog by Tween 20 is histamine, and they offer suggestive evidence for the ability of cortisone to influence histamine metabolism.

Material and methods. Male dogs were used. Two injections of Tween 20† were given intravenously at either 24 or 48 hour intervals under pentobarbital anesthesia. The dose of Tween 20 was 0.1 ml per kg of a 5% solution in water. Arterial blood pressure was recorded from the femoral artery with a mercury manometer. Ten dogs received 4-7 mg of cortisone‡ per kg of body weight intramuscularly every 6 hours day and night. Cortisone treatment was begun 6 hours prior to the first Tween injection and was continued throughout the experiment for 24 or 48 hours. Eleven dogs received the Tween injections without cortisone treatment and served as controls. Histamine in the plasma was demonstrated by paper chromatography. The method described by Urbach(14) with the extraction procedure of McIntire(15) was used with the modification of employing ascending instead of descending chromatography. Blood was drawn immediately before and 5 minutes after the injection of Tween. Citrate was the anticoagulant used. Twenty ml of plasma was the usual volume for the extraction of histamine. Known quantities of histamine were added to dog's plasma. These plasma samples were extracted simultaneously with the unknowns and the final butanol extracts were chromatographed on the same sheet of filter paper.

Results. A. Demonstration of histamine in dog's plasma following the injection of Tween 20. When dog's plasma obtained 5 minutes after the injection of Tween 20 was analyzed for histamine by paper chromatography, it produced a spot of the same Rf value and the same color as plasma samples to which known amounts of histamine were added. This fact has been demonstrated conclusively in 8 separate experiments. The paper chromatographic technic is not suitable for the exact quantitation of the amount of histamine released by Tween 20. Nevertheless, certain conclusions may be drawn from these studies. In some experiments, in which a 25% solution of Tween 20 was injected intravenously, the amount of histamine found in 20 ml of plasma was greater than 10 μg. In the majority of the experiments, the quantity of histamine was estimated to lie between 5 and 10 μg per 20 ml of plasma.

B. Effect of cortisone on the Tween reaction. Table I shows the effect of 2 injections of Tween 20 on the mean blood pressure of male dogs. The first injection produced a marked fall of blood pressure. The second injection, given 24 or 48 hours later, produced a lesser but still significant fall of blood pressure in all untreated control animals. Cortisone had no effect on the response to the first Tween injection. It decreased or prevented the response to the second injection. The differences between cortisone treated and untreated male dogs appear to be striking

† Tween 20 was obtained from the Atlas Powder Co., Wilmington, Del.

‡ Cortone Acetate Merck.

TABLE I. Blood Pressure Changes in Cortisone-Treated and Untreated Male Dogs Following Two I.V. Injections of Tween 20.

Dog No.	Treatment	Intervals, hr	Tween injections: First (mm of Hg) Initial	5 min	10 min	Second (mm of Hg) Initial	5 min	10 min
1	None	24	130	42	42	125	98	75
2			125	41	42	132	92	86
3			122	42	33	110	65	42
4			113	42	40	110	85	96
5			115	41	42	119	87	82
6			110	50	52	108	62	52
7			108	48	63	92	47	52
	Mean ± S.D.		118 (8.1)	44 (3.7)	45 (9.7)	114 (13)	77 (18.7)	69 (20.1)
8	None	48	130	48	32	122	70	80
9			125	50	61	92	60	73
10			120	50	51	128	75	90
11			115	60	65	115	95	106
	Mean ± S.D.		122 (6.5)	52 (5.4)	52 (14.7)	114 (15.8)	75 (14.7)	87 (14.3)
12	Cortisone	24	150	55	50	100	60	84
13			144	65	64	110	127	98
14			123	45	45	126	115	124
15			120	50	52	120	105	115
16			120	50	56	95	96	98
17			120	43	47	114	82	70
18			105	50	57	90	90	89
	Mean ± S.D.		126 (15.6)	51 (7.3)	53 (6.5)	108 (12.5)	96 (22.1)	97 (18.3)
19	Cortisone	48	162	58	57	140	140	138
20			138	86	62	120	141	150
21			130	58	75	115	119	119
22			120	56	70	110	118	130
	Mean ± S.D.		137 (17.9)	64 (14.4)	66 (8	121 (13.2)	129 (12.7)	134 (13.1)

when the second injection of Tween 20 was administered 48 hours after the first injection. Under these conditions, the 4 cortisone treated dogs were completely refractory to the hypotensive action of the second injection of Tween 20. In 2 of these dogs the blood pressure actually rose.

In addition to these experiments, 2 male dogs received cortisone treatment in the usual way for 24 hours before receiving the injection of Tween 20. Cortisone again failed to prevent the usual response of dogs to the first injection of Tween.

Three of the dogs which were refractory to the second injection of Tween 20 as a result of treatment with cortisone received an intravenous injection of histamine (10 μg per kg). They responded with a marked fall of blood pressure to the injection of histamine. Fig. 1 shows the blood pressure tracing of one of these experiments. The cortisone treated dog responded to the injection of histamine, whereas it failed to respond to the second injection of Tween 20.

A limited number of experiments were performed on female dogs. Cortisone treatment produced complete refractoriness to the second injection of Tween 20 in only one out of 5 female dogs. These experiments suggest that it may be more difficult to demonstrate the interference of cortisone with the Tween phenomenon in female dogs.

Discussion. Since our paper chromatographic studies show that Tween 20 liberates histamine in the dog, the interference of cortisone with the hypotensive effect of a second injection of Tween 20 is probably related to an effect of cortisone on the production, destruction or release of histamine. It is obvious that cortisone does not interfere with the release of preformed histamine, since it does not protect against the first injection of Tween 20. It is also obvious that cortisone does not protect against the effects of re-

FIG. 1. Effect of inj. of Tween 20 and of histamine on mean blood pressure of a dog treated with cortisone. At A, Tween 20 was inj. At B, inj. was repeated 24 hr after the first inj. At C, histamine was inj. Time in min.

leased histamine. The most likely possibilities are that cortisone prevents the resynthesis of histamine in the tissues following its release and or it stimulates the destruction of histamine in the tissues. Either one of these hypotheses would explain why the second injection of Tween 20 fails to produce hypotension.

The effect of cortisone on Tween hypersensitivity may be looked upon as a prolongation of the refractory period which according to Krantz and coworkers(12) lasts 14 to 16 hours. Unfortunately the nature of the refractory period is not well understood. Krantz suggests that the refractory period represents the time required for the destruction of Tween. If this hypothesis were substantiated by experimental data, it could be argued that cortisone influences the Tween phenomenon by delaying the destruction of the compound.

There are no data in the literature concerning the breakdown of Tween 20 in the dog. Experiments on the destruction of this compound by isolated rat tissues(16) indicate that Tween 20 is broken down by active esterases which are present in most tissues. It is difficult to believe that cortisone could prevent the destruction or excretion of Tween 20 for 48 hours in the dog, and it is very unlikely that our data could be explained on that basis. The failure of cortisone to prevent anaphylaxis in the guinea pig, where histamine release is quite certain, is comparable with the failure of cortisone to prevent the response of dogs to the first injection of Tween. It should be of interest to study the effect of cortisone on repeated anaphylactic shocks in the guinea pig and such experiments are in progress.

The results of this study fit in best with the hypothesis that cortisone is capable of influencing histamine metabolism in the dog in such a way that it prevents the accumulation of histamine in the tissues once they are depleted of that compound by the action of Tween 20. Only further studies will show whether or not the observations and interpretations of the present study may contribute to the understanding of the mechanism of action of cortisone in hypersensitive states.

Conclusions. (1) The injection of Tween 20 in the dog is followed by the appearance of histamine in the plasma, as demonstrated by paper chromatography. (2) Cortisone does not prevent the hypotensive effect of the first injection of Tween 20, in the male dog. (3) Cortisone prevents or decreases the hypotensive effect of a second injection of Tween 20 administered 24 or 48 hours after the first injection. (4) Cortisone does not prevent the hypotensive effect of injected histamine. (5) It is postulated that cortisone prevents the accumulation of histamine in the tissues following the release of this compound by Tween 20.

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A Hitherto Unrecognized Factor Against Dietary Necrotic Liver Degeneration in American Yeast (Factor 3) (19240)

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Most American yeasts, in contrast to many European yeasts, do not induce necrotic liver degeneration when used as protein source in purified, vit. E free rations for rats. However, the deficiency can be obtained readily with American grown torula yeast(1), and it can be prevented either by cystine or by vit. E (2). In earlier work some evidence had been accumulated for the involvement of a third unidentified factor in the prevention of the defect(3). It had been noted particularly that the addition of a few percent crude casein, or ordinary "vitamin free", alcohol extracted casein to necrosis producing diets inhibited the damage,* whereas purified, alkali-treated casein "Hammarsten" did not influence the development and had even been used extensively as a main dietary component for the production of the deficiency(4).

It is shown in the following experiments that American yeasts G† and K,‡ which do not produce the defect(1) have a considerable protective activity when added in small amounts to torula diets. It is concluded from simple fractionation experiments with yeast K that this effect is not due to the presence of vit. E or cystine but that protection is brought about by a third, unidentified principle which has been designated "factor 3."

Experimental. Details concerning the induction of the deficiency have been described (1). Weanling Sprague-Dawley rats of the National Institutes of Health strain were used exclusively. Animals in this series were males except for a few groups of mixed sex as indicated. Each group started with 10 animals. Groups in one experiment consisted of equally distributed litter-mates of even average weight. The basal ration, as reported previously, contained 30% torula yeast, 59% sucrose, 5% salts, 5% vit. free lard and a vitamin mixture(1). From this formula the different experimental diets were derived by adding supplements at the expense of torula or of sucrose. Tests for protective activity were performed in two ways: (A) Prophylactic method (Table I, exp. A, B and C). The supplement was given from the beginning of the experiment. (B) Repletion method (Table II). The animals were kept on the basal diet, in sets of 5 litter mates to a cage, for a preliminary period of 23 days. At the 24th day they were separated and supplementation was started. The repletion method was adopted as a standard procedure, since it requires less space and less supplemented material and yields results in a shorter experimental time. It is based on two observations: Very few rats succumb to dietary necrotic

* To be published separately.

† Cultured, primary dried yeast, Anheuser-Busch, Inc., St. Louis, Mo.

‡ Debittered, dried brewer's yeast, Anheuser-Busch, Inc.

TABLE I. Different Levels of Torula Yeast and Inhibitory Effect of Yeast G and Yeast K on Dietary Necrotic Liver Degeneration (Prophylactic Method).

Exp.	Torula yeast, %	Yeast G, %	Yeast K, %	No. of animals	Dead due to liver degeneration	Avg of reciprocal survival times (×100)
A (different levels of torula yeast)						
	20	0	0	8	8	2.20§ ± .15‡
	30	0	0	8	8	2.43 ± .25
	40	0	0	8	8	2.15 ± .13
B*	30	0	0	9	9	2.48 ± .10
	30	0	0	6	6	1.87 ± .25
	30	10	0	9	3	.66 ± .25
	30	0	10	9	0	.0 ± .0
C†	20	0	0	10	10	2.22 ± .04
	20	0	10	10	1	.13 ± .12

* Terminated after 130 days. † Terminated after 90 days. ‡ Stand. dev. of mean. § Avg survival time = 47 days.

TABLE II. Inhibitory Effect of Yeast K, and Fractions Thereof (Repletion Method).

Exp.	Torula yeast, %	Supplement, %	No. of animals	Dead due to liver degeneration	Avg of reciprocal survival times (×100)
D*	30	—	9	9	2.40 ± .18‡
	27	Yeast K 3	10	2	.58 ± .35
	25	" 5	8	0	.0 ± .0
E†	30	—	9	9	2.17 ± .19
	30	" extract** ≅ 5	9	8	2.00 ± .32
	30	" residue** ≅ 5	10	1	.21 ± .23
F*	30§	—	9	9	2.40 ± .18
	25	" residue** ≅ 5	10	2	.54 ± .32
	25	Residue hydrolysate†† ≅ 5	10	1	.19 ± .18

* Terminated after 72 days. † Terminated after 75 days. ‡ Stand. dev. of mean. § Same control group as in Exp. D.

** One part yeast K boiled for 20 min with 5 parts of water, filtered by suction, procedure repeated. The residue was extracted twice by boiling 20 min with alcohol. Water and alcohol extracts combined, evaporated to dryness and dissolved in 2 parts water: "yeast K extracts." Residue of extraction dried at air until free from alcohol: "yeast K residue."

†† One part of "yeast K residue" hydrolyzed by boiling with 10 parts of 4 N sulfuric acid 18 hr, SO_4 was removed by Ba hydroxide. Ba sulfate filtered off and washed 3 times with boiling water. Combined filtrates evaporated to dryness, taken up in 2 parts of hot water and stored at +4°C several days. Precipitate formed, containing some cystine, removed by filtration. Filtrate, consisting of soluble portion of hydrolysate, was "residue hydrolysate."

liver degeneration before the twenty-fourth day, and substances active against the defect, like vit. E, afford protection even when supplementation is started at this time(5). For evaluation of the results the average of the reciprocal of the survival times was calculated for each experimental group and compared to littermate controls(1).

Results. Variation of dietary levels of torula yeast. The influence of various levels of torula yeast on the development of dietary necrotic liver degeneration was studied, since different dietary levels of this material were involved in some of the experiments, and also because the question has been raised as to whether the presence of a "toxic" substance in some yeasts, and its absence in others, would explain the differences between yeasts of various sources in this respect(6,7). There was no significant difference between groups with 20, 30, or 40% torula in the diet (Table I, exp. A, see also controls in exp. B-F).

Inhibition by yeast G and yeast K, prophylactic method. On the 30% torula ration the incidence of dietary necrotic liver degeneration was 100%, the usual average survival time was 45.5 days(1). When 10% yeast G was added from the beginning of the experiment, 6 out of 9 animals survived (Table I, Exp. B). A more pronounced effect

was caused by 10% yeast K, which afforded complete protection. Survivors of the experiment were killed after 130 days; their livers were found to be normal. When 10% yeast K§ was added to the 20% torula diet (Table I, Exp. C) only one animal died, at the 78th day, and the other 9 displayed normal livers after 90 days. A control group on the 20% torula ration died from liver degeneration within the usual length of time.

Inhibition by yeast K, repletion method. Subsequent tests showed that almost complete protection against dietary necrotic liver degeneration was provided when 3% or 5% of yeast K was added at the expense of torula, beginning on the 24th day, after a depletion period of 23 days on the standard torula ration (Table II, Exp. D).

Elimination of cystine and vit. E. For reasons which have been discussed extensively by various authors attempting to interpret the difference between necrosis inducing yeasts and others(7-9) the protective efficacy of yeasts G and K could hardly be ascribed to cystine or tocopherol, the known protective agents. The amount of cystine in yeast is small. Cystine determinations were performed in the protein fraction obtained after removal of interfering substances by extraction with boiling water and alcohol, using the method of Sullivan *et al.*(10). After hydrolysis .6% cystine was found in the torula protein fraction, the corresponding values for yeast G and K were .7% and .6%. Replacement of a small fraction of torula yeast in the diet by yeast G or yeast K protein therefore causes no significant change in the overall cystine content. Tocopherol does not seem to be a normal component of dried brewer's yeast which has been used extensively as a standard ingredient of vitamin E free rations (11-13). Customary chemical assay methods can not be applied to yeast since yeast fat contains interfering substances(5).

The fact that the protective quality of yeast G and K could not be attributed to cystine or vit. E was established by fractionation of the active principle from yeast K.

§ The following description is restricted to experiments with yeast K, the more effective material.

TABLE III. Substances Inactive Against Dietary Necrotic Liver Degeneration Induced by Torula Diet (Prophylactic Method).

Supplement	Doses*		No. of rats	Dead due to necr. l.d.
Thiamine	4	mg %	10†	10
Riboflavin	25		10†	10
Pyridoxine	20		10†	10
Nicotinamide	20		10†	10
Ca pantothenate	4		10	10
Inositol	10		10†	10
Choline chloride	100		10	10
Biotin	10	γ %	10†	10
Folic acid	100		10†	10
	100	γ d.s.‖	10†	10
Vit. B_{12}	.1		6†	6
	[illegible]		9	9
Vit. B_{12} + folic acid	.25+100		8	8
Citr. factor‡	2		9	9
Citr. factor§	2		9	9
Vit. K	.9	mg %	10	10
Ascorbic acid	.5	%	10†	10
Xanthine	.5		10†	10
Alpha-amino ethanol	.5		9	9

* % means % in diet. † Group contained 50% females. ‡ "Leucovorin" Lederle, kindly supplied by Dr. E. L. R. Stokstad, Lederle Laboratories, Pearl River, N. Y. § Started on 8th day of experiment. ‖ d.s. = daily subcutaneously.

Fractionation experiments with yeast K. A simple procedure was devised which would separate cystine, and also tocopherol if present. Yeast K when boiled twice with water and twice with ethanol left an insoluble residue amounting to 60 to 65% of the starting material. This consisted mainly of protein. Tocopherol and its esters would have been removed by the alcohol extraction. The protein residue had the full protective activity of yeast K (Table II, exp. E & F). This material was hydrolyzed with hydrochloric or with sulfuric acid. After neutralization and concentration of the hydrolysate, a little cystine and considerable tyrosine precipitated out. The remaining solution did not give a detectable reaction for cystine, which indicated that the cystine content was less than .1% of the dry weight. The preparation had full activity against dietary necrotic liver degeneration when added to torula diets in an amount equivalent to 5% yeast K.

Ineffective substances. The commonly known vitamins and some other substances

were tested against dietary necrotic liver degeneration and had no appreciable activity (Table III). In some of these tests the supplement seemed to shorten the life span, in others a slight prolongation of the survival time could be observed. However, in all of these groups no animal survived. It seems noteworthy that xanthine which is known to protect against chloroform liver damage(14), and which was found to inhibit dietary liver degeneration caused by casein VI diets(4), had no activity. This might be explained by the presence of high amounts of nucleic acid in yeast which furnish an appreciable quantity of xanthine during the metabolic breakdown of adenine and guanine. Alpha-amino ethanol, which protects against L-penicillamine intoxication(15), was also ineffective.

Discussion. The results of this investigation demonstrate clearly that yeast G and especially yeast K exert a strong protective effect against dietary necrotic liver degeneration when added in relatively small amounts to torula diets. It is evident that this activity is due to a substance (or substances) different from vit. E and from cystine. The possibility has to be left open that factor 3 is an active derivative of either cystine or of tocopherol. Factor 3 is water-soluble and stable against acid hydrolysis. In yeast it is firmly bound, presumably to protein. It is not identical with any of the better known water soluble vitamins. Whether factor 3 is an ordinary non-catalytic building stone of cell matter or whether it is of vitamin nature remains to be settled.

Dietary necrotic liver degeneration was originally thought to be a cystine deficiency (16-18). When the preventive role of vitamin E was elucidated(19) it became evident that, at least on yeast diets, either cystine or vitamin E alone prevented the damage, and it was concluded that necrotic liver degeneration in this instance was due to a simultaneous deficit in both of these factors. The dietary interchangeability was interpreted as indicating a metabolic interrelation between these substances(2). This conception can now be extended; under our experimental conditions a simultaneous lack of 3 factors—cystine, vit. E and factor 3—is a prerequisite for the development of dietary necrotic liver degeneration, and each one of them alone can protect.

The possibility has been discussed that a hypothetical toxic component, which would have to be present in some yeasts and absent in others, could be the reason for the discrepancy between yeasts of different degeneration-inducing capacities(6,7). Evidence in favor of this postulate could not be detected in the present experiments. The deficiency can be induced by a large variety of different rations without any yeast, as pointed out before(1), and the speed of the development of the liver damage does not increase with increasing dietary levels of torula. The discovery of factor 3 in yeasts which do not induce the damage provides a natural and adequate explanation for the differences in regard to this deficiency.

Dietary insufficiency of vit. E can manifest itself by a great variety of pathological lesions according to species and to dietary conditions used (general edema or fatal encephalomalacia in chicken, muscle dystrophy in guinea pigs, rabbits, goats, calves, and also ducks and turkeys, necrotic liver degeneration or general edema or slowly developing genital lesions in the rat, etc.)‖ The question arises whether different levels of factor 3 in various rations are the reason for the multiplicity of these vit. E deficiency diseases, since it appears from a comparison of the results of this investigation with earlier work that the appearance of the avitaminosis in rats is decisively influenced by the absence or presence of this factor, dietary necrotic liver degeneration killing the animal within several weeks in the first case, the disturbance of gestative functions developing more slowly in the second.

Summary. (1) It is shown that yeast G (cultured, primary dried yeast) and yeast K

‖ These defects, including dietary necrotic liver degeneration, are much more severe and probably more specific than the slowly developing, well-known deficiency symptoms in the genital tract. Tocopherol should not be considered to be merely an antisterility factor, its functions being essential for brain, liver, muscles, including the heart, and probably for other organs.

(debittered, dried brewers yeast), which do not induce liver degeneration, exert a strong protective influence when added to diets causing the disease. It has been demonstrated by fractionation experiments that this effect is not due to vit. E or cystine, but due to a third unidentified factor, designated factor 3, which has been concentrated from yeast K. (2) The presence of factor 3 explains why dietary necrotic liver degeneration cannot be produced with American yeasts G and K. Factor 3 is water soluble, stable against acid hydrolysis, and is not identical with any one of the presently well-known vitamins.

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Free Amino-Acids in Rat Brain During Insulin Shock. (19241)

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It has been reported(1) that after insulin administration, the rat's brain content of free glutamic acid lessens, free amino acid nitrogen is slightly diminished, but no changes occur either in glutamine or ammonia. The explanation suggested was that during the severe conditions accompanying hypoglycemia, glutamic acid is oxidized, and that ammonia is in some way metabolized or liberated from the brain, without any change taking place in other free amino acids. Nevertheless, the authors considered it necessary to submit this last assertion to the test of experiment, particularly in connection with amino acids closely related to glutamic acid in tissue metabolism, such as aspartic and γ-aminobutyric acids, the latter recently found in brain as a decarboxylation product of glutamic acid(2,3).

Experimental. Nine groups, each with 6 to 8 young male albino rats each weighing 100 to 150 g were used. Rats of groups 1-7 received no food (water *ad libitum*) during 24 hours, and then, to half the rats, subgroup B, 350 IU/k of Lilly's insulin were injected intraperitoneally, the other subgroup A, being kept as controls. Starvation conditions were further maintained, in the subsequent period, during which insulin shock developed in subgroups B, and some of their members died. Rats of groups 8 and 9 were not starved before their subgroups B were injected with 60 IU/k insulin, thus maintaining them under conditions similar to those in Dawson's ex-

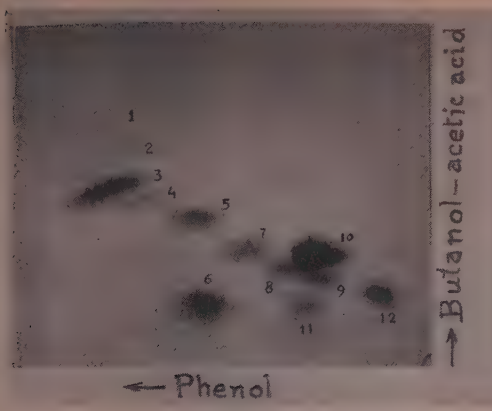

FIG. 1

FIG. 2

FIG. 1 and 2. Two chromatograms from rat brain extracts, normal (1) and during insulin shock (2). Spots numbered: 1, leucine and isoleucine; 2, valine; 3, γ-amino-butyric acid; 5, alanine; 6, glutamine; 7, threonine; 8, glycine; 9, serine; 10, glutamic acid; 12, aspartic acid. 4 and 11, unidentified.

periments(1). At the end of the 4 hour period for groups 1-7, and only 2 hours for groups 8 and 9, rats still alive were killed by cervical vertebral dislocation. The brains from members of each subgroup were immediately dissected, pooled, and homogenized in a Waring Blendor with 80% ethyl alcohol. The resulting suspensions, after treatment by Awapara's method(4), furnished protein and lipid-free extracts, some of which were desalted by electrodialysis in an apparatus assembled on the principles followed by Consden *et al.*(5). After a 10-fold concentration, by boiling, extracts with 200-400 μg of amino acids/0.01-0.02 ml were finally used for running threefold series of chromatograms on Whatman No. 1 filter paper, by the bi-dimensional descending technic of Consden *et al.* (6), in the phenol-80% butanol-acetic acid-water (4:1:1) system. Filter paper sheets were dried in a current of heated air, and sprayed with a dilute solution of ninhydrin in butanol. *Rf* values for the spots of glutamic, aspartic and γ-amino-butyric acids, and glutamine, were checked by running chromatograms of standard solutions of amino acids, as well as by adding them to brain extracts. Concentrations of each amino acid and glutamine in the spots were measured by the colorimetric method of Naftalin(7) in a Coleman Junior spectrophotometer, at 570 mμ, glutamic acid being used as a standard for glutamine. Total amino acid nitrogen was measured in duplicate samples of some of the extracts, by Sahyun's colorimetric method(8). All steps were carried out simultaneously for brains from paired A and B subgroups.

Results and discussion. Inspection of Fig. 1 and 2, and of Table I, shows that brain extracts of insulin injected rats have, as compared with those from untreated animals, lesser amounts of free glutamic acid, γ-amino-butyric acid and glutamine, and conversely, a greatly increased content of free aspartic acid and practically no change in the free amino acid nitrogen level.

On the whole, our results are in agreement with those of Dawson regarding glutamic acid and amino acid nitrogen, but introduce the new finding of the increased free aspartic acid, which requires an equally new explanation.

Contraposition of such noticeable increase in free aspartic acid with the decrease in glutamic acid level, associated with the lack of important changes in total amino acid nitrogen, suggests that under the experimental conditions, the increase in aspartic acid may be due to activation of a transamination reaction between glutamic and oxalo-acetic acids. α-ketoglutaric acid, another product of the same reaction, probably increases under the emergency conditions accompanying hypoglycemia and enters the tricarboxylic acid cycle. The great importance of such a transamina-

TABLE I. Free Amino Acids, Total Amino Acid Nitrogen and Glutamine in Brain of Rats, Normal and During Insulin Shock.

Groups	Hr of starving	IU/k	mg/100 g brain tissue: Total amino acid, N A	Total amino acid, N B	Glutamic acid A	Glutamic acid B	Aspartic acid A	Aspartic acid B	γ-amino-butyric acid A	γ-amino-butyric acid B	Glutamine A	Glutamine B
1	24	350	—	—	168.4	127.9	37	110	29.1	20.6	43.7	42.2
2	24	350	—	—	166.6	124.1	52.8	161.7	43.4	32.7	40.5	13.5
3	48	350	39.6	33.9	173.1	127	40	146.3	30.9	17.3	31.6	11.7
4	24	350	—	—	166	77	42.7	84.4	44.8	22.4	33.5	7
5	24	350	37.2	29.9	194.7	136.4	44.8	66	30.1	22.5	41.4	9
6	24	350	45	44.8	146.3	88.9	34.3	138.6	31.7	19.5	34.5	6.5
7	24	350	33	30.5	188	150.7	42.7	55.9	24.7	16.2	—	—
8	0	60	31	30.5	160.9	147.4	41.6	58	38.9	30.6	32.4	17
9	0	60	30.8	30.1	189.2	166.1	40.1	81.2	41.6	25	—	—

A, control rats; B, rats under insulin shock.

tion system in normal brain tissue, has been pointed out by Cohen and Hekhuis(9).

Increased activity in transamination between glutamic and α-ketoglutaric acids explains, furthermore, why the ammonia level remains practically unaffected during insulin hypoglycemia, and makes the lowering of γ-amino-butyric acid and glutamine levels, referable to diminution in available free glutamic acid.

Summary. Brain extracts of rats subjected to insulin shock have, as compared with those from normal animals, smaller amounts of free glutamic acid, γ-amino butyric acid and glutamine, and a greatly increased quantity of free aspartic acid.

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Effect of Vitamin E and CCL_4 on Fat, Respiration and Choline Oxidase of Rat Livers.* (19242)

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A previous report(1) indicated lower liver fat in young rats deficient in vitamin E as compared with controls receiving this factor. On a diet low in protein and deficient in vitamin E, young rats are known(2,3) to be highly sensitive to acute CCl_4 toxicity; this condition is corrected by supplements of alpha tocopherol. CCl_4 is known to produce an immediate fatty infiltration of the liver, and Ennor(4) has shown an increased rate of respiration of liver slices from guinea pigs poisoned with CCl_4. Therefore, the influence of vitamin E on the response of liver fat and the rate of liver slice respiration after acute CCl_4 intoxication has been investigated in rats.

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TABLE I. Liver Fat of Rats as Influenced by Vitamin E, Protein Level and CCl_4.

CCl_4/wk, ml	Dietary casein level, %	Dietary α-tocopheryl acetate, %	No. of rats	Avg 8 wk wt gain, g	Liver fat* (dry basis) %	S.E.	t
0	10	0	12†	93	8.7	± .493	5.23§
0	10	.01	12	112	12.7	± .586	
0	18	0	5	213	9.4	± .789	.78
0	18	.01	5	219	8.6	± .618	
.05	10	0	6‡	71	20.1	±1.30	.81
.05	10	.01	8	101	18.2	±2.12	
.05	18	0	5	219	23.8	±2.16	.77
.05	18	.01	5	227	21.2	±2.63	

* Obtained 24 hr after final CCl_4 inj.
† 16 rats started; 4 died with typical vit. E lung hemorrhage syndrome(1).
‡ 12 rats started; 6 died with typical vit. E lung hemorrhage syndrome(1).
§ By the Fisher *t* test with 22 degrees of freedom a P value of 2.4 indicates significance at the 1% level.

Data on the effect of dietary vit. B_{12} have been included since, following acute CCl_4 poisoning, Popper, Koch-Weser and Szento (5) reported that this factor protected against liver damage and Hove and Hardin(3) reported protection against fatality in rats. Choline oxidase has been estimated on homogenates from livers of rats receiving various dietary supplements as well as CCl_4, since this enzyme system possibly is related to fat metabolism. Other work from this laboratory (6) showed that CCl_4 poisoning inhibited the methylase system in liver as measured by creatine synthesis. It is of interest to determine whether this was a general effect on liver enzymes.

Methods and results. The basal diet contained water-washed casein(7) 10%, sucrose 76%, lard 9%, cod liver oil 1%, and salt mixture(7) 4%. Sufficient dry vitamins were ground with a portion of sucrose and added to the diets to furnish the following levels per gram: thiamine, riboflavin and pyridoxine, 5 μg each; calcium pantothenate, 25 μg; niacin, 4 μg; choline chloride, 2 mg; i-inositol, 0.2 mg; and 2-methyl 1-4 naphthoquinone, 2 μg. Variations in this diet were made in a few cases in which the casein was raised to 18%, or the lard was raised to 19%, or both, at the expense of the sucrose level. Supplements to the diets were as follows: none; alpha tocopherol acetate 0.01%; vitamin B_{12} 30 μg/kg (as a concentrate); or both of these factors. Male albino rats (Sprague-Dawley) were placed on the diets at weaning (40 to 50 g), housed individually, and food and water supplied, *ad libitum*. For the animals reported in Table I, the time on experiment was 8 weeks. During this time about half of the animals on each treatment received weekly subcutaneous injections of 0.05 ml of CCl_4 diluted 1:1 in olive oil. Twenty-four hours after the last injection, these, as well as the uninjected rats, were killed for liver fat determination. After removal, the livers were dried overnight in an air oven at 105°C, ground, and analyzed for ether extractable material under code numbers.

Liver fat of rats on the 10% casein diet and not receiving vit. E was distinctly and significantly lower than that of controls receiving this factor (Table I). However, no difference was noted due to the vitamin E supplement when the diet contained 18% casein. Following the CCl_4 injections, the liver fat increased sharply to about the same degree on all diet treatments.

The rats reported in Fig. 1 had been on the diets for 5 weeks prior to injection with 1 ml of CCl_4/kg body weight. At this time they weighed 95 ± 11 g. Two rats from each diet treatment were removed at time intervals of 0, 1, 2, 4, and 8 days after the injections. The livers were analyzed for fat, and respiration of liver slices was determined as follows: About 200 mg of fresh liver slices obtained with the Stadie slicer were dropped into Warburg vessels containing 3 ml of glucose-Ringers phosphate solution(8). The rate of respira-

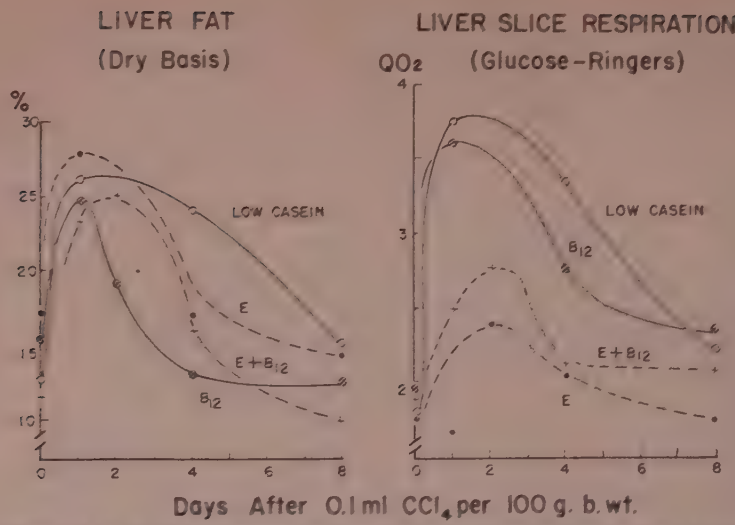

FIG. 1. Effect of single CCl_4 injections on liver fat and on rate of respiration of liver slice as influenced by dietary supplements to the 10% casein diet (low casein) of α-tocopheryl acetate (E), vit. B_{12} 3 μg/kg (B_{12}), and the combination of these vitamins (E + B_{12}). Each value represents average for 2 rats that had been on the diets for 5 weeks prior to injection. Fat values are expressed on the dry basis and respiration values as mm³ O_2 uptake per hr per mg of dry fat-free liver, with 2 mg glucose in 3 ml of Ringer's phosphate.

tion at 37.5 C was expressed as the oxygen consumed per hour per mg of dry, fat-free tissue.

From the data in Fig. 1, it is evident that the vit. E and B_{12} supplements had no marked influence on the course of fatty changes in the livers of rats 24 hours after the injections of CCl_4. There was indication that the supplements, especially vit. B_{12}, allowed a more rapid return to normal liver fat values.

The respiration of liver slices was increased following the injections of CCl_4, especially when vit. E was not included in the diet. Dietary supplements of vit. B_{12} had no effect on this phenomenon (Fig. 1).

The choline oxidase values reported in Table II were determined on livers of rats that had been on the indicated diets for periods of 6 to 12 weeks. The livers were removed 24 hours after the rats were injected, i.p., with 2 ml CCl_4 per kg body weight. One ml of the homogenized liver (1:3 in phosphate buffer, pH 7.4) was added to Warburg vessels containing 2 ml of buffer, or 2 ml of buffer with 4 mg of choline chloride. The difference in oxygen uptake between the flasks with and without choline was considered to be a measure of the choline oxidase activity of the liver.

The data in Table II indicate that the choline oxidase activity of the liver was not influenced by dietary tocopherol supplements or by CCl_4 poisoning. However, an increased level of fat in the diet appeared to increase the choline oxidase activity.

Discussion. Liver fat of young rats on a vit. E deficient 10% casein diet was increased 4 percentage points, from 8.7 to 12.7, by inclusion of alpha tocopheryl acetate in the diet, a highly significant difference statistically. This result confirms previous data(1) in which the average liver fat of rats, similarly treated except for a higher fat level in the diet, increased from 12.3% to 14.2% (dry basis), and is in accord with the general conclusion of Menschik(9) that a vit. E deficiency in mice resulted in a marked disappearance of both liver and body fat. The level of dietary fat may have a bearing on the absolute values of liver or body fat found since in the previous work, and for the data of Fig. 1, the diet contained 20% fat as compared with 10% in the diet of rats reported in Table I.

TABLE II. Choline Oxidase of Rat Liver Homogenates. Rats had been on diets 6 to 12 weeks from weaning.

Single CCl_4 inj., ml/kg	Levels/100 g diet			No. of rats	mm^3 O_2/hr/mg dry liver*		
	Casein, g	Fat, g	α-tocopheryl acetate, g		Choline/flask— None	4 mg	Increase due to choline
0	10	10	0	8	.46	1.92	1.46
0	10	10	.01	7	.32	1.76	1.44
0	10	20	0	8	.90	3.45	2.55
0	10	20	.01	4	.61	3.34	2.73
0	20	20	0	5	.95	3.89	2.94
0	20	20	.01	3	1.33	3.47	2.14
2	10	10	0	2	1.10	2.67	1.57
2	10	10	.01	2	.80	2.18	1.37
2	20	20	0	4	.63	3.38	2.75

* 24 hr after CCl_4.

The increased liver slice respiration after CCl_4 treatment confirms Ennor's work(4) on guinea pigs. Since in the present study the increased rate of respiration parallels the increased fat content of the liver, it is possible that this fat is being catalytically oxidased, and that the tocopherol when fed, inhibits this oxidation by simple antioxidant action.

It is clear that the striking sensitivity to acute CCl_4 toxicity displayed by young rats on 10% protein diets deficient in vitamin E, and preventable by vitamin E, vit. B_{12} or a higher casein level(2,3,10), cannot be attributed to an altered course of fatty changes in the liver following CCl_4.

Summary. (1) Liver fat of young rats on a vit. E-deficient, 10% casein diet was increased 4 percentage points, from 8.7 to 12.7, by the inclusion of alpha tocopheryl acetate in the diet, a highly significant difference statistically. When the diet contained 18% casein, no difference was noted due to supplements of alpha tocopheryl acetate. (2) Dietary treatments did not influence the increase in liver fat 24 hours after CCl_4 injections, though vit. B_{12} supplements resulted in more rapid return to normal values. The rate of respiration of liver slice in glucose-Ringers was doubled following CCl_4 injections. This was substantially inhibited by dietary supplements of vit. E. (3) Choline oxidase activity of the liver was not influenced by dietary vit. E or by CCl_4 injections into rats, although increased dietary fat level increased the activity of this enzyme system.

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Comparison of Canine Gastric Juice with 0.1 N HCl as Stimulus for External Pancreatic Secretion. (19243)

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Unpublished experiments in this laboratory have suggested that commercial preparations of gastric mucus as well as commercial pepsin have a slight stimulating effect on the pancreas when placed in the intestine. The results were inconclusive but seemed worthy of further exploration. Experiments were, therefore, undertaken to determine whether gastric juice, as secreted, contains any pancreatic stimulus other than HCl.

Methods. Three mongrel dogs were used weighing 15 to 25 kg and equipped with gastric and duodenal cannulae as described by Thomas and Crider(1). The pancreatic duct was cannulated through the duodenal fistula as described by Hart and Thomas(2). The gastric juice was obtained from the same or other similarly equipped dogs after the subcutaneous injection of 1.0 mg of histamine base or the intravenous injection of 15 to 20 units of insulin. The juice was filtered through gauze and the free and total acidity determined by titration with 0.1 N sodium hydroxide using Toepfer's Reagent and Phenophthalein as indicators. Values for free and total acidity were so nearly the same that it was apparent that it would make no significant difference which value was used in estimating the volume of gastric juice equivalent in acid value to a given volume of HCl. Ten cc of 0.1 N HCl and equivalent amounts of gastric juice in terms of total acidity were compared as stimuli for the pancreas. In each experiment 4 injections of one of the test materials were made at 10-minute intervals, followed by 4 injections of the other. Reversal of the order made no difference in the results. Injections were made through a tube which was passed into the duodenal fistula until its tip lay approximately 6 inches distal to the fistula. The pancreatic juice obtained after the first stimulus with any test solution was discarded. The volume and specific gravity (determined gravimetrically) of each remaining sample of pancreatic juice were recorded.

Results. Table I shows the number of injections of each test solution made and the mean values for volume and specific gravity of the pancreatic juice secreted in response to the specified stimulus. Although the individual volumes varied widely, there is no significant difference in the mean volumes secreted in response to hydrochloric acid, gastric juice secreted in response to insulin and that in response to histamine. The values for specific gravity were less variable and in 2 of the 3

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TABLE I. Mean Volume and Specific Gravity of Pancreatic Juice Secreted in Response to .1 N HCl and to Gastric Juice.

Dog	Stimulus	No. of samples	Vol, cc	Specific gravity
1	10 cc .1 N HCl	17	7.3 ± 2.3	1.011 ± .0008
	Histamine gastric juice†	20	8.2 ± 2.9	1.011 ± .0007
	Insulin gastric juice†	5	7 ± .9	1.011 ± .0004
2	10 cc .1 N HCl	23	7.2 ± 3.8	1.012 ± .0008
	Histamine gastric juice†	9	10.9 ± 4.8	1.012 ± .0008
	Insulin gastric juice†	15	7.5 ± 2.2	1.012 ± .001
3	10 cc .1 N HCl	13	3.4 ± 1.1	1.012 ± .003*
	Histamine gastric juice†	13	4.2 ± .8	1.011 ± .0008
	Insulin gastric juice†	17	4.1 ± .9	1.012 ± .003*

* A single determination of specific gravity in each group of 1.021 and 1.024 respectively accounts for the large stand. dev. On both occasions secretion of pancreatic juice eventually ceased and in one instance a mucous plug was actually demonstrated obstructing the cannula.

† Volume of gastric juice used ranged from 6.5 to 12.8 cc.

dogs the mean value is the same for all 3 stimuli. In the third the difference was not significant.

Comment. The specific gravity of pancreatic juice has been used in this laboratory as a measure of the nitrogen output(3) and, by inference, the protein output of the pancreas(4). The latter, in turn, may be taken as an index of the output of enzymes(5-7). Our results indicate that canine gastric juice secreted in response to either histamine or insulin contains no substance other than HCl which modifies either the volume or the nitrogen content of pancreatic juice. Probably the slight stimulating effect of some commercial preparations of substances normally found in gastric juice is due to contaminants.

Conclusion. Canine gastric juice contains no stimulus for the pancreas other than HCl, which can be revealed by our method of study.

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Hypoproteinemia as Protection in Mercuric Chloride Poisoning. (19244)

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Certain experimental states apparently protect animals from $HgCl_2$ toxicity. A hemoglobinuric dog survived 4 mg $HgCl_2$/kg(1), albuminuric rats survived toxic doses of organic mercurials(2), and hypoproteinemic dogs survived 3 mg $HgCl_2$/kg(3). A possible mechanism for the latter action is indicated by the present experiments.

Experimental. Two normal dogs fed a 6% protein 10:3:3 crackermeal:sugar:lard diet supplying 75 Cal./kg/day were plasmapheresed daily by replacement of 25% estimated blood volume by an equal volume of a 50% dog erythrocyte suspension in 0.9% NaCl, until plasma protein totaled less than 4.4 g %. With 5 untreated normal dogs as controls, all dogs were given 3 mg $HgCl_2$/kg intravenously, except one hypoproteinemic dog who received 4 mg/kg. Daily urine Hg^{++} content(4) and clinical signs were noted. To urine samples having less than 0.001 mg Hg^{++}/ml, the lower limit of 3% accuracy for this method, .002 mg $HgCl_2$ was added per ml. Table I shows probable error limits of each determination. Surviving (hypoproteinemic) dogs were then put on a stock diet of 1/4 pound raw horse meat daily plus Friskie dog chow *ad lib.*, and 3 additional doses of 3 mg/kg of $HgCl_2$ were given 1, 2, and 6½ months after the initial dose. Except for omission of urinalysis after the fourth dose, procedure was the same each time.

Results. The 4 control dogs whose plasma protein level exceeded 6.1 g % died 2½ to 4½ days after the $HgCl_2$ dose. The fifth control dog had 5.8 g % plasma protein and died in 16½ days. Each had anorexia, weakness, emesis, bloody diarrhea, ulcerative stomatitis, initial diuresis, albumin- and hemoglobinuria and, in the 12-hour period before death, hyperventilation with rates up to 240/min., weak and rapid pulse and tetany of facial, neck and shoulder muscles. Dehydration, fatty liver, noncrepitant lungs, hemorrhagic intestinal mucosa, and necrosis and edema of renal cortices were seen at autopsy. No control dog excreted as much as half the $HgCl_2$.

TABLE 1. Cumulative % Excretion of Mercuric Chloride.

Days after dose	Control dogs					Initially hypoproteinemic dogs				
						First dose	Second dose		Third dose	
	1 (6.5)*	2 (6.1)	3 (7.4)	4 (6.3)	5 (5.8)	1 (4.4)	2 (4.2)	1 (7.7)	2 (5.7)	1 (6.9)	2 (6)
1	11.4	26	38.1	45.8	36.3	47.5	42.7	48.8	39.6	60.3	49.7
2	12.7	28.7	38.4 (3)†	46.1 (21)	37.6 (8)	55.6	55.9	54.4	47	66.7 (4)	55
3	12.9 (7)	28.9 (5)	38.7 (10)	46.3 (13)	38.1 (9)			57	50.4	70.2	59.1
4		29.3 (8)	38.9 (6)		38.3 (17)	78.7	62.3	57.6 (6)	52.3 (5)	72.5 (3)	61.4
5		30 (7)			38.4 (30)	89.1	65.9	58.8	53.6 (4)	73.9	63.7
6					38.6 (14)	93.4	68.7	59.4 (7)	54.3 (5)		
7					38.7 (21)	94.6	70.2	60.2 (6)	54.8 (14)	74.8 (44)	63.8 (37)

* Figures in parentheses indicate g % total plasma protein at time of $HgCl_2$ inj.
† " " " probable error of determination of Hg++ excreted when probable error is over 3% in that 24 hr period, assuming probable error of original method to be no more than 3%.

The 2 hypoproteinemic dogs showed essentially no toxicity. Stomatitis, bloody diarrhea and fever were seen only within the 4 days after the second and third doses. In each case, each dog excreted more than half the Hg^{++} within 3 days. The fourth dose, half a year after the initial hypoproteinemia, caused the characteristic response to the poison, terminating in death within 5 days.

Discussion. Hypoproteinemia is characterized by a relatively large and prolonged albumin deficit, albumin restoration lagging well behind the osmotically less active globulin. The lowered colloidal osmotic pressure allows increased extracellular volume(5) and therefore a greater dilution of the poison. Probably more important, mercury is partially bound to plasma albumin and the per cent bound is less when the albumin:mercury ratio is low (6). "Free Hg^{++}" is greatest with low plasma albumin; this is assumed to be the basis for the increased excretion rate over controls in our experiments. Since return of albumin to normal electrophoretic pattern takes over 3 times as long as the return of total albumin concentration, and requires many weeks when protein intake is only moderate(7), protection from Hg^{++} toxicity would last even longer if Hg^{++} is bound primarily to a slowly-returning fraction. So far there is no independent evidence for the last possibility, but the effects of total albumin level may be crucial. This may explain the finding that hypoproteinemic dog 1 was protected from the second dose, with a plasma protein level of 7.7 g %, and was killed by the fourth dose 5½ months later, with a plasma protein level of 6.7 g %. The possibility exists that changes in the pattern of renal and other intracellular proteins on protein depletion may alter the susceptibility of the cells to the toxic effects. Since there are no data on this point, further speculation does not seem profitable.

Summary. Five normal and 2 hypoproteinemic dogs were given similar intravenous $HgCl_2$ injections. Each normal dog died with characteristic mercury poisoning signs; the hypoproteinemic dogs survived this and 2 more such doses, time interval between doses being 1 month, but died from a fourth dose

given 6½ months after the initial hypoproteinemia, at which time their plasma protein levels were 6.7 and 5.8 g %. Control dogs excreted less than half the mercury; the hypoproteinemic dogs excreted over half of it within the first 3 days. It is suggested that the resistance to mercury toxicity seen in the hypoproteinemic animals may be due to increased extracellular volume and, more important, a relatively small binding of Hg++ by protein, permitting more rapid excretion and diminished cell susceptibility.

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Effect of Intravenous Injections of Desoxycorticosterone Glucoside upon Blood Glucose of Adrenalectomized Dogs.* (19245)

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It is well established that the oil soluble preparation of DCA, given intramuscularly in small doses, exerts little, if any, effect upon the intermediary metabolism of carbohydrate. On the other hand, those adrenal steroids oxygenated at C-11 are highly efficacious in this respect(1-5). However, within recent years several reports have appeared indicating that DCA, administered in large doses, induces glycosuria in the force-fed, partially depancreatized rat(6) and "restores glycogen production to normal from carbohydrate or protein in liver and muscle of adrenalectomized animals which are kept alive for some time" (7,8). Harrison and Harrison(9) concluded from their experiments that "the injection of adequate amounts of desoxycorticosterone acetate into fasted adrenalectomized rats does prevent the drop in concentration of blood sugar found in untreated adrenalectomized rats." Other investigators have reported some positive effects of large amounts of DCA on gluconeogenesis and blood sugar level of adrenalectomized rats(10) and the glucose requirements of adrenalectomized-eviscerated rats(11). In the course of a study on the efficiency of the water soluble glucoside of desoxycorticosterone‡ (DCG), in reviving adrenalectomized dogs from insufficiency, massive doses of this steroid were administered as a single injection by vein. Since the amounts employed were far larger than anything reported in the literature, and since fasted adrenalectomized animals receiving DCG, during revival from severe insufficiency, were only occasionally observed to exhibit sharp increases in blood glucose, it was considered worth while to study in more detail the possible correlation between DCG and blood glucose levels.

The present report is based upon a study of 16 adrenalectomized dogs and one intact animal. The type of dog employed, amount of DCG administered and other details of the

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TABLE I. The effect of Massive Doses of DCG on Fasted Intact and Adrenalectomized Dogs.

Dog No.	Period of fasting, hr Pre-inj.	Post-inj.	Initial B. P., mm Hg	DCG dosage, mg	Blood glucose, mg % Pre-inj. period	Post-inj. period 3 hr	6 hr	12 hr	18 hr	24 hr
				Intact animal						
1	30	24	110	300	66	76	70.5	72.5	—	67.5
				Adrenalectomized in normal health						
2	30	24	103	300	62.5	72	70.5	62	—	66.5
3	30	24	115	600	68	69	66	66.5	—	68.5
4	30	24	105	600	66	68.5	72	71.5	—	66.5
				Adrenalectomized in mild insufficiency						
5	30	24	70	300	69	70.5	76	73.5*	71	72.5
6	30	24	71	300	79.5	76.5	81.5	—	80.5	87.5
				Adrenalectomized in severe insufficiency						
7	30	24	50	300	72.5	81.5	90	—	119.5	111.5
8	30	24	55	300	61.5	88	88.5	84 *	84	81
9	30	24	54	300	85	62.5	71.5	72.5	69	75
10	30	24	52	300†	64	65	62.5	74	72	76
11	30	24	46	300	64	66	—	65	70	65

* 16 hr sample. † 200 mg DCG 24 hr later.

experiment concerned solely with fasted animals, are shown in Table I. In order to eliminate exogenous sources of carbohydrate, all food was withheld for 30 hours prior to the initial injection of DCG and for 24 hours thereafter. Thus, the dogs were subjected to a prolonged fast of 54 hours. This is severe treatment for adrenalectomized animals maintained solely on a steroid presumably without effect upon gluconeogenesis. The experimental results are presented in Table I.

Examination of the data reveals that of the 11 fasted dogs, receiving a single minimum dose of 300 mg of DCG intravenously, only 2 animals (No. 7 and 8) showed marked increases in blood glucose. These dogs exhibited severe insufficiency symptoms, such as, low arterial pressure of 50 and 55 mm Hg, spasticity and muscular weakness at the time DCG was injected. The blood glucose of one animal (No. 7) rose from the pre-injection level of 72.5 mg to 119.5 mg % during an 18-hour interval. The second dog (No. 8) showed an elevation in glucose from 61.5 mg to 84 mg % during the same time interval.

Three other adrenalectomized animals with equally severe symptoms (No. 9, 10 and 11) revealed either slight or no changes in blood glucose following injection. The maximum increase observed in these dogs, during the 24-hour observation period, was 12 mg % (dog 10). This animal had received a total of 500 mg of DCG in 2 injections: 300 mg at the time of crisis and 200 mg 24 hours later. The post-injection observation period for this animal was extended an additional 12 hours with no further significant alterations in the blood glucose level.

The other types of animal used in this experiment, *i.e.*, intact and adrenalectomized, but maintained free from symptoms of insufficiency, and those dogs, presenting only mild symptoms (Table I), failed to show any appreciable or sustained rise in blood glucose, following DCG treatment. It seemed evident that injections of massive doses of the glucoside by vein to fasted, adrenalectomized dogs is without effect upon the blood sugar of most of the animals. An occasional dog suffering from severe insufficiency may exhibit a significant rise in blood glucose following DCG treatment. However, the writers do not attribute the sharp increases in glucose in these exceptional cases to an effect of the DCG *per se*, but consider the sugar elevation to be due to the marked general improvement of the animal's condition, *e.g.*, the increased hydration, increased blood flow through organs and tissues, and opening up of previously closed capillary beds. This interpretation of the effects of DCG upon the blood glucose of adrenalectomized dogs is in line with a suggestion originally made by Russell(11) some years ago to account for

TABLE II. Effect of Massive Doses of DCG on Non-Fasted Adrenalectomized Dogs in Insufficiency.

Dog No.		Adrenal insufficiency	24 hr	48 hr
1	B. P., mm Hg	50	57	80
	Glucose, mg %	72	87.5	89.5
	DCG dosage, mg	300	200	100
2	B. P., mm Hg	54	86	89
	Glucose, mg %	66	83	88
	DCG dosage, mg	250	100	100
3	B. P., mm Hg	59	62	72
	Glucose, mg %	67.5	86	88.7
	DCG dosage, mg	300	200	50
4	B. P., mm Hg	65	67	87
	Glucose, mg %	82	99.5	110
	DCG dosage, mg	300	200	200
5	B. P., mm Hg	78	84	98
	Glucose, mg %	76.5	80	93
	DCG dosage, mg	300	150	50
6	B. P., mm Hg	78	91	114
	Glucose, mg %	76	75	80
	DCG dosage, mg	300	150	50

certain apparent effects of DCA upon glucose utilization of her adrenalectomized-eviscerated rats.

In contrast to the equivocal results obtained by administering DCG to fasted adrenalectomized dogs, are the blood sugar changes which follow such injections in the non-fasted animal exhibiting insufficiency. During the first 24-48 hours of the revival period, the dogs reveal striking and unmistakable elevations in blood glucose. The arterial pressure(12) and blood sugar record of 6 such animals are recorded in Table II.

The dogs were permitted to develop mild to severe symptoms of adrenal insufficiency. None of the animals had eaten food for approximately 12 hours before the initial blood samples were taken. Examination of the blood pressure data will give some idea of the severity of the symptoms presented by these animals at the beginning of the experiment.

Immediately following injection of DCG, the usual diet was given and at the end of 24 hours the blood pressure and blood samples were taken. As shown in Table II, a significant elevation of the blood glucose occurred in 5 of the 6 animals. There was no direct correlation between the rise in arterial pressure and the increased blood glucose. One dog showed a sharp rise in blood pressure but a negligible change in the sugar level (No. 6, Table II) at the end of the first 24 hours. Other dogs (No. 2 and 4) revealed rises in sugar and also elevations in the arterial pressure. As a general rule, however, the blood pressure does not return to normal levels before 72-96 hours after injection. The dogs listed in Table II were eager for food and displayed great improvement in activity and vigor in spite of the still lowered blood pressure. The marked effect of DCG upon the blood sugar in these cases was probably due to the increased appetite and improved alimentary absorption of glucose, and not to any direct action of the steroid upon carbohydrate metabolism.

Summary and conclusion. (A) Three types of fasted adrenalectomized dogs were studied: (1) those maintained in normal health; (2) those exhibiting mild insufficiency symptoms; and (3) animals in severe adrenal crisis. When these dogs were injected intravenously with massive doses of DCG, only occasional blood glucose changes were observed. These infrequent rises in glucose were associated with marked improvement in the circulation of the animal, increased hydration, increased vigor and muscular activity. (B) A fasted, intact dog, injected with 300 mg DCG, did not show any significant changes in blood sugar level over a 24-hour period. (C) Adrenalectomized dogs, presenting severe symptoms but allowed food *ad libitum* during revival from insufficiency, exhibit sharply increased blood glucose levels in the recovery period following DCG administration.

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Augmented Excretion of Urine Gonadotrophins During ACTH Administration. (19246)

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Observations on the effects of ACTH on renal excretion of pituitary gonadotrophin have been made by the author during the past two years. Recently, Sohval and Soffer(1) reported the results of a similar investigation in 22 patients receiving ACTH and/or cortisone. They called attention to the limited data on the subject, specifically those of Mason *et al.*(2) and Sprague *et al.*(3), who observed no change in urinary gonadotrophin titers of 2 young women. Sohval and Soffer (1) found enhanced titers in 9 patients of their series but they stated that the mechanism of this change remained unknown. The present report concerns the results of gonadotrophin analysis of 73 urine samples from 9 patients receiving ACTH. Methods of gonadotrophin isolation and bioassay were employed which differed from those used in the above-mentioned investigations.

Materials and methods. Pertinent data on the case material is presented in Table I where dosage, route and duration of the hormone administration are summarized with the gonadotrophin titers. All patients received ACTH and Case 5 received, in addition, a short course of oral cortisone therapy. Case 3 received 15 mg of ACTH intravenously over a 6-hour period while all others received the hormone intramuscularly in divided doses. Urine samples, collected over periods of from 12 to 24 hours, were preserved under toluene. Gonadotrophins were isolated by adsorption on activated kaolin(4). The gonadotrophins were dried under a stream of nitrogen, sealed in a test tube and kept at 3°C until assayed. At this time the powdered material was dissolved in isotonic saline and the bioassay carried out as reported by Lloyd *et al.*(5). This method employs 21-day-old female white mice of 8 to 10 g, using 2 mice at each of 4 dilutions per gonadotrophin sample. Reassay at the same or different dilutions was carried out when confirmation was desired or the end point was not obtained initially. In these instances, a total of 16 mice per sample were required. Volumes of 0.25 cc of gonadotrophin solution were injected subcutaneously twice daily for 3 days and the animals sacrificed 24 hours following the last injection. Throughout the assay period the solutions of gonadotrophins were kept at 3°C. Both ovarian and uterine weights were recorded but only the latter were used to determine the titers. Mice were assayed in lots of 50 of which 5 to 7 served as saline-injected controls. A positive result was recorded when the average of the uterine weights of the 2 gonadotrophin-injected animals at any level exceeded the average of the saline-injected controls by 100%. Toxicity of the gonadotrophin solutions was encountered infrequently and only 9 of more than 800 mice used in the study were lost. One group of 5 mice received a total of 4.1 mg of ACTH per mouse in 6 divided doses over a 3-day period to determine the direct effects of this hormone on the uterine, ovarian and adrenal weights (Table II).

Results. In all cases augmented gonadotrophin excretion of varying degree was observed during the period of ACTH adminis-

TABLE I. Clinical Data, ACTH Dosage* and Urinary Gonadotrophin Levels.†

Case	Age, sex	Gonadal status	Day of treatment 0	1	2	3	4	5	6	7	8	9	10	11	15	22
1	63 ♂	Normal								>16			>16			
			<8	<8	8	32			8	<32			<32	16	16	8
				65	75	60	50	40	50	60	50	40	40	40	20	0
2	57 ♂	”	16	32	32	16	16	16	8	<8	<8					
				80	80	50	40	40	35	10	0					
3	44 ♀	Regular menses	8	16	16											
				15§	0											
4	17 ♀	Primary amenorrhea	<8	<4	12	<4	<4	<4	6	<4	<4		<4			
				40	40	40	40	40	40	0	0	0	0			
5	79 ♀	Postmenopausal	—			> 64		>32			>16					
				32	32	<128	64	<64	64	32	<24	24	24			
				100	100	100	100	100	75	50	150‡	150‡	150‡			
6 (a)	63 ♀	”				>256										
			64	128	256	<512	256	64	<64		128	128		64		
				70	60	50	40	30	30	22.5	7.5	0	0	0		
(b)	—	—	—	>128				> 64								
				<256	64	512	512	<128				64				
				40	70	60	60	50	20	10	0	0				
7	50 ♀	Menopausal	—	> 64												
				<128	128	256	64									
				20	80	45	0									
8	54 ♂	Normal	4	16	16	16		<4	8							
				80	70	40	30	10	0							
9	32 ♀	Irregular menses	<8	64				64			8			8		
				100	100	100	75	75	75	75	75	50	0	0		

* Lower figure for each day is the total ACTH in mg per 24 hr.
† Upper ” ” ” ”; expressed in mouse units per 24 hr.
‡ Oral cortisone acetate in mg per 24 hr.
§ Intravenous ACTH.
< No assay performed at lower dilution.
/ Increase in average of uterine weights over controls approached 100% at the higher dilution.

TABLE II. Comparison of Body, Uterine, Ovarian and Adrenal Weights of Gonadotrophin, ACTH and Saline-Injected Mice.

Group	No. of mice	Mean body wt	Mean uterine wt	Mean ovarian wt	Mean adrenal wt
Saline-inj.	9	11.2 g	15.6 mg	2 mg	2 mg
Gonadotrophin-inj.	12	11.6	55.8	4.8	2.4
ACTH-inj. (4.1 mg total)	5	11.2	18.2	2.5	3.6

tration. In Case 4, however, an increased titer was found only on the second day of treatment and, thereafter, the level did not exceed the control. Although a 2-fold increase in titer was observed in Case 3, the control urine in this instance was collected 6 days prior to the ACTH administration. Lack of a control urine in Case 5 prevents an interpretation of the magnitude of increase that can be attributed to the effects of the corticotrophin. Highest titers in this patient were observed, however, from the 3rd through the 6th day of therapy when ACTH dosage was maximal, while the lowest titers were obtained during the period of oral cortisone administration. Exitus of the patient during cortisone therapy prevented a post-treatment control observation. In Case 7, therapy was instituted 4 hours prior to completion of the first day of urine collection and this specimen, for the most part, may be considered as a control. Two to 8-fold increases were observed in the remaining patients, the greatest in Case 6 and 9. Changes were often abrupt as demonstrated by the increased titers of Cases 2, 6, 8 and 9 during the first 24 hours of therapy and by the decreases in Cases 1, 2, 7 and 8 when the dosage was reduced or the hormone discontinued. In Case 6(a), however, higher titers returned when ACTH was discontinued and for at least 24 hours thereafter. In general, highest titers were found during periods of most intensive hormonal therapy, but no close correlation to ACTH dosage can be made. Where positive results were obtained using uterine weights, similar interpretations could be made from ovarian weight increases which, in the lower dilutions of the gonadotrophin solution, often were marked. The ovaries, if enlarged, characteristically were pale and occasionally ovarian hemorrhages and hyperemia were observed only at the lower dilutions. In the mice receiving ACTH (Table II), ovarian and uterine weights did not differ enough from the controls to be considered positive by the criteria above mentioned, yet the adrenal weights nearly doubled. In 12 gonadotrophin-injected animals chosen at random among positive reactors, marked ovarian and uterine weight increases were found while adrenal weights were increased slightly.

Discussion. The results presented here are supplementary to those which have been reported recently by Sohval and Soffer(1). The fact that the methods of gonadotrophin isolation and bioassay have differed in the two studies adds further significance. The extent of the increases in the excretion of gonadotrophins in the two series is similar as the above investigators(1) found changes of approximately 2 to 10-fold. Whereas they demonstrated the gonadotrophic nature of the substance by failing to obtain uterine weight increases in ovariectomized mice, the present investigator has achieved a similar result by observing significant increases in ovarian weights. That the predominant gonadotrophin was FSH is suggested by the observation that the enlarged ovaries, with few exceptions, were pale. Ovarian hyperemia and hemorrhagic corpora lutea were seen only as isolated occurrences at low dilutions of the injected material. The above mentioned investigators made early observations in only one patient who was found to have a titer of 180 mouse units on the second day of ACTH therapy. They were unable to determine the duration of the augmented excretion primarily due to the scatter of specimens assayed and the frequent toxicity of the preparations injected. In the present series, 4 (Cases 2, 6, 8 and 9) were found to have their control titers at least doubled during the first 24 hours of hormonal therapy. Whether increased levels continued throughout therapy appears to have

been more a matter of a critical dosage than the duration of treatment, as suggested in Cases 2, 5, 6 and 8. Discrepancies appeared, however, in that Case 6(a) was found to have enhanced titers 24 hours following ACTH withdrawal and Case 9 was observed to have an 8-fold reduction in titer during 4 days of a fixed ACTH dosage. The possibility of a pituitary rebound effect in the former instance might be considered. With these irregularities and the known limitations of bioassay, it would be inappropriate to suggest a quantitative relationship between dosage and gonadotrophin excretion. It is apparent that similar gonadotrophin responses were elicited in male and female patients and that the degree of augmentation was not related to the gonadal status or the initial level of gonadotrophin excretion. This suggests that the explanation for the enhanced titers is less likely to be found in direct hormonal interrelationships than in a possible renotrophic effect of the adrenal steroids. Significant increases in glomerular filtration following ACTH administration have been reported(6-8). These changes, approximating 30%, would not account for the 2 to 8-fold increases in gonadotrophin titers recorded here and the 2 to 10-fold increases previously reported(1). Increased proteinuria has been observed during ACTH administration(9) and enhanced glomerular permeability might account for additional renal loss of the trophic hormones.

The observations on the dwarf with intrasella calcification (Case 4) suggest that increased pituitary function is essential for the maintenance of the enhanced titers. From clinical study and from an evaluation of 4 months of vaginal smears, the patient was considered to have incomplete sexual maturation with no significant variations in the low level of ovarian function. With a calcified anterior hypophysis and a fixed release of gonadotrophins (less than 4 m.u.) she was unable to sustain the transient response to the renotrophic effects of ACTH by releasing more gonadotrophin from the pituitary. It is questionable whether the results in Case 3 can be attributed to ACTH. The control urine specimen was collected 6 days prior to the day of intravenous ACTH which was administered on the 12th day of the menstrual cycle.

Absence of significant changes in ovarian and uterine weight of intact mice receiving ACTH in large amounts (Table II), essentially excludes the possibility that recovery from the urine of this hormone was partly responsible for the enhanced gonadotrophin titers during its administration. There is some evidence, furthermore, that the kidney is unimportant in the disappearance of ACTH from body fluids(10,11).

Further data are necessary before results herein presented can contribute to an understanding of the aberrations of gonadal function which have been observed during or following intensive ACTH therapy. It is possible, however, that increased renal loss of FSH during ACTH therapy might divert enough trophic hormone from the gonads to disturb their function. The possibility is recognized that a similar increased excretion of other trophic hormones might lead to a temporary aberration of end-organ function, such as the ACTH-induced hypothyroidism previously reported(12,13).

Summary. Augmented excretion of urine pituitary gonadotrophins has been demonstrated in 8 of 9 patients receiving ACTH for the treatment of several clinical entities. In the one exception, the doubling of the control gonadotrophin titer could not be attributed directly to the effects of the ACTH. Increases of 2 to 8-fold were observed in the remaining patients. The augmentation appeared to be independent of age and sex, was not proportional to the dose of ACTH, and showed irregularities in time of appearance or persistence. In the patient with hypophyseal calcification, the effect was observed on the second day of therapy only which suggested that normal hypophyseal function was essential for the increased excretion. The known renotropic effect of the adrenal steroids was considered the most probable explanation for the observed changes.

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Effects of Growth Inhibitors on Response of Rat's Uterus to Estrogen.* (19247)

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Hertz and Sebrell(1) first reported the failure of estrogens to induce growth of the female reproductive tract in the absence of folic acid in the chick, and subsequent studies followed on the frog(2), rat(3), and monkey (4,5). In all of these studies the main objective was centered around gross morphological observations and weight relationships. The experiments reported here represent an attempt to examine the physiological nature of the inhibitory process produced by folic acid antagonists on the growth response of the uterus to estrogen, and to determine in what way this may differ from similar inhibitory effects produced by other compounds such as beryllium(6), cadmium(7), and strychnine. These comparisons are made on the basis of inhibitory effects on the imbibition of water by the uterine tissue in response to estrogen and changes in nitrogen, fat, and glycogen content.

Materials and methods. A total of 56 100-day-old female albino rats, of an inbred strain developed from Wistar stock, weighing 175-200 g, were used for these experiments. A folic acid antagonist (Aminopterin†), and beryllium, cadmium, and strychnine were given concurrently with estradiol.‡ The estradiol was dissolved in sesame oil, and the other drugs in physiological saline. All injections were made subcutaneously and the dosage of beryllium, cadmium, and strychnine was such that the animals given such treatment would survive for at least two weeks without loss of weight. The animals given aminopterin for 3 to 4 days lost little or no weight. Nitrogen determinations were made colorimetrically(8), while the tests for fat and glycogen were made by histochemical

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† Aminopterin(4-aminopteroylglutamic acid) was obtained through the courtesy of Lederle Laboratories, Pearl River, N. Y.

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TABLE I. Effect of a Folic Acid Antagonist and Beryllium, Cadmium, and Strychnine on Water Content and Total Nitrogen of Uteri of Ovariectomized Rats Given Estrogen.

Group	Treatment	Uterine wt, mg	% H_2O	N, μg/mg
I	——	89.7	79.6	23.2
II	.1 μg est.*	190	80.9	24.5
III	.1 μg est. + 50 μg A5040†	129.3	78.4	22.5
IV	.1 μg est. + 50 μg A9332	132.7	78	23.4
V	.1 μg est. + 30 μg A5801	162	80.3	21.7
VI	.1 μg est. + .1 mg Be./g B.W.‡	167.8	77.8	23.5
VII	.1 μg est. + .01 mg Cd./g B.W.	147.6	79.7	24.2
VIII	.1 μg est. + 1 mg strychnine/kg B.W.	129.1	78.9	25.6

* Estradiol-17-β. † A = Aminopterin (4-aminopteroylglutamic acid). ‡ B.W. = Body wt.

procedures(9,10). The animals were ovariectomized and divided into 8 groups at the very beginning of these experiments. The animals of Group I served as castration controls, and were killed 11 days after the operation. The Group II animals, 7 days after castration, were given 0.1 μg estradiol daily for 3 days. The remaining groups, III to VIII, were put on experiment after the uteri had undergone one week of castration atrophy. On the seventh day of this period a single dose of the inhibitor was given, and the treatment was continued for 3 additional days during which both the inhibitor and estradiol were given concurrently. The animals were killed 24 hours after the last injection. The uteri were removed and slit lengthwise and blotted on bibulous paper thus removing excess fluid, and weighed separately on a Roller-Smith torsion balance.

Results and discussion. All animals treated with aminopterin, beryllium, cadmium, and strychnine showed uteri that were much smaller than those given estradiol alone. There was also a decrease in percentage of nitrogen in the uteri of those receiving the folic acid inhibitor. Aminopterin 5040 and 9332, in equivalent doses, produced a decrease of 8.1 and 4.5% respectively, while 5801, which was more toxic, produced a decrease of 11.4% at three-fifths the dose of the other two. This is in sharp contrast with the results for controls given estrogen alone. In these there was an increase of 5.6% in uterine nitrogen (Table I).

A decrease in the total nitrogen content of the uteri was also obtained in those animals receiving beryllium and cadmium, 4.1 and 1.2% respectively. On the other hand, strychnine did not effect a decrease in the nitrogen content of the uterus, but on the contrary was associated with an increase of 4.3%. Therefore, strychnine has a very weak inhibitory action on the effect of estrogen.

The only animals that showed a positive test for fat were the untreated castrate controls. This is in agreement with the observations of Dietlein(11) who has found that estrogen does not only fail to bring about the deposition of fat in the endometrium of the rat's uterus but removes any that may be present in the luminal epithelium. Thus, it appears that in these experiments sufficient estrogen remained not inhibited to remove the fat that was presumably present in the luminal epithelium of the castrated animal.

Glycogen was absent from the uteri of both experimental and control animals. This was checked both by colorimetric(12) and histochemical methods(10). Likewise, there was a uniformity of results with regard to the imbibition of water by the uterus in that each inhibitor produced a decrease as compared with controls given estrogen alone.

Summary. Aminopterin, beryllium, cadmium, and strychnine proved effective in reducing the physiological activity of estradiol. All compounds except strychnine effected a decrease in total nitrogen content in the uteri of ovariectomized rats receiving estradiol. Such inhibition was associated with a decrease of water in the uterine tissue and the absence of fat and glycogen. Thus, the inhibitory process is associated with these physiological changes.

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An Influence of 2,6-Diaminopurine upon the Content of Kappa in *Paramecium aurelia,* Variety 4.* (19248)

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Many compounds which, from their structure, might be expected to interfere with nucleic acid metabolism, have been tested for their ability to restrain the growth of neoplasms in experimental animals(1-7). The studies are based upon an assumption that the metabolism, the pattern of uptake of precursors of nucleic acid in normal cells, is different from that in neoplastic ones. It is assumed, furthermore, that the differences are sufficiently important to permit their use in inducing by anti-metabolites the selective injury of the neoplastic cell type, as compared with the normal. This approach to cancer chemotherapy has the advantage, in theory, of being applicable whether the neoplastic process be due to a virus or to a mutated genic structure, since both are dependent for function on their component nucleic acids. Of the compounds which have been synthesized and tested as potential antimetabolites of nucleic acid, few have exerted a high degree of growth restraint of any type of neoplasm *in vivo.* None have been curative. Nevertheless, *in vitro,* a substantial degree of selective injury to some, but not all, neoplastic cells, as compared with normal types growing at the same rate and of generally similar origin, has been demonstrable (8).

The possibility of differential toxicity based upon the presumed specificity of nucleic acid metabolism of different type cells is attractive and important. Accordingly, new means were sought to provide evidence pertinent to this assumption. The characteristics of the cytoplasmic factor kappa in the ciliated protozoan, *Paramecium aurelia,* as elucidated by Sonneborn and his associates(9-18), indicated that it might provide a useful subject for study. Certain stocks of *P. aurelia,* known as killers, contain a sufficient concentration of kappa particles to produce paramecin, a substance lethal to other stocks of the protozoan. The susceptible animals, known as sensitives, contain no kappa particles and form no paramecin. X-ray inactivation studies and staining procedures indicate that the kappa particle is roughly comparable in size to certain viruses and rickettsia. Kappa particles are self-duplicating at rates independent of the fission rate of the paramecia containing them, and contain desoxyribonucleoprotein(15,16), as does their product, paramecin(17). Could kappa, an easily demonstrable and functionally unique cell constituent, be destroyed with-

*We wish to acknowledge support of this study by funds from the American Cancer Society.

out injury to its host, strong evidence for a high degree of specificity of the nucleic acid anti-metabolite would be at hand. This would lend support to further studies of compounds with similar action for their chemotherapeutic value in cancer control.

Method. At 27°C, and with an abundant supply of nutrients, kappa in variety 4, stock 51 killer paramecia can duplicate at approximately the same rate as the animals containing it. At 33.8°C(11), however, kappa particles are maintained without increase in number, while paramecia continue to reproduce. Thus, kappa may be so dispersed that many organisms will contain no kappa, and as a result, not only lose their ability to produce paramecin, but also become sensitive. Kappa in animals with a partially depleted supply may be built up to a higher concentration by returning them to 27°C and reducing their reproduction to 1 fission per day by limitation of food supply. Such animals will again produce paramecin when a sufficient kappa concentration has been restored. These methods have been applied in the expansion technic(11,14,18) for quantitative determination of kappa particles present in paramecia subjected to conditions which might decrease the amount of kappa. A nitrogen mustard, HN2, (methyl bis(beta-chlorethyl)amine), has been shown to inactivate kappa(18). Compounds of that type are highly reactive and may exert their effects by combination with a number of chemical groups and at many sites in a molecule(19). Hence, their mechanism of action is difficult to ascertain. HN2 is reactive for such a limited time, furthermore, that the reported experiments with it required modification before they could serve as models for tests of materials such as potential anti-metabolites of nucleic acid.

A screening procedure utilizing the killer-sensitive test(11) was employed to detect the capacity of test substances to affect significantly the concentration of kappa and the production of paramecin. Test compounds were dissolved in a mixture of 1 part baked lettuce culture medium(11) and 3 parts salt solution(20) in concentrations of 100, 200, 500, 1000, and 1500 μg/ml at an adjusted pH of 6.4 to 7.0. Ten to twenty non-autogamous, semi-starved killers (stock 51, mating type VII, variety 4), were exposed in a 0.2 ml volume of each concentration for 24 hours at 27°C. For controls, an identical group of killers was incubated under the same conditions in a 0.2 ml volume of the culture medium-salt solution mixture. Three paramecia were then isolated from each of the two highest concentrations of the compound which the animals survived. Similar isolations were made from the control cultures. The isolated organisms were placed in fresh culture medium and allowed to reproduce at maximum rate for 48 hours, at which time they had undergone 9 or 10 fissions. Killer-sensitive tests were then made on the clones from the isolated animals; *i.e.*, they were tested for their ability to kill sensitives and also for their susceptibility to killers. A clone control without added test animals indicated whether autolethality was present. After incubation for 24 hours at 27°C, the tests were observed for lethal action. It was found that the progeny of killers exposed to 2,6-diaminopurine† in concentrations of 500 μg/ml were markedly weakened in killing ability and that some had become sensitive, as indicated by autolethality in the clone control and susceptibility to test killers. Observations at 48 hours indicated that as the animals became more starved under the test conditions, thereby decreasing their fission rate, the ability to kill was strengthened.

To obtain quantitative information on the decrease in effective kappa resulting from exposure to 2,6-diaminopurine, data were sought through the expansion technic(11). The observations indicate a decrease in paramecin-producing kappa to 5-10% of the level in untreated animals(24). Many other purines and pyrimidines, including 8-aza-adenine, 8-azaguanine, and substituted 2,6-diaminopurines were tested and none have exerted an effect similar to 2,6-diaminopurine. These experiments will be reported later(21).

Since the ability of natural purines to block or reverse the inhibitory effects of 2,6-diamino-

† We are indebted to Dr. George H. Hitchings, Wellcome Research Laboratories, Tuckahoe, N. Y., for supplying this material.

purine in other biological systems has been reported(22-23), studies of such possibility in the case of kappa were made. Concentrations of test purine in 0, 5, 10, 25, 50, 100, 250, and 500 μg/ml were used in a mixture with 500 μg/ml of 2,6-diaminopurine. After a 24-hour exposure, isolations and killer-sensitive tests were run as previously described. Adenine, adenosine, and adenylic acid were found to block the effect of 2,6-diaminopurine on kappa(21).

Additional evidence is being sought through staining procedures, electron microscopy, and radioautography on the question of whether the amount of effective kappa is reduced by 2,6-diaminopurine through prevention of its formation, through production of an abnormal kappa, or by its actual destruction.

Summary. The content of effective kappa in *Paramecium aurelia* killers is markedly reduced by their exposure to 2,6-diaminopurine. This purine uniquely exhibits this capacity among the many purines and pyrimidines tested. The effect is blocked by the simultaneous presence of adenine, adenosine, or adenylic acid.

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Detection of Norepinephrine in the Parotid Gland Secretion of *Bufo agua*.* (19249)

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The presence of epinephrine in the parotid gland secretions of various species of toads has

* This species of toad is known by various names. *Bufo agua* and *Bufo marinus* have been most frequently used. The "glands" referred to are aggregates of glands behind each ear, and are usually designated as either "parotid" or "parotoid". The term parotid is used here in its literal sense since in structure, function, and content the toad gland described above is completely different from the salivary gland which is commonly associated with the term.

been known for many years(1-5). Recent investigations have suggested the frequent coexistence of norepinephrine and epinephrine in animal tissues, with detectable amounts of both having been reported in the adrenal gland, chromaffine tumors, sympathetic nerves, and most organs(6). Bacq and LeComte, however, found no trace of norepinephrine in the secretion of *Bufo arenarum* (7). Fischer and LeComte, working with *Bufo marinus,* could detect norepinephrine in but one parotid gland out of 54 studied, but noted that in each assay on the cocaine-sensitized nictitating membrane, the response was greater than that expected from the epinephrine content as determined by a fluorometric method(8). Recently, Lee and Chen have reported the presence of small quantities of norepinephrine in an extract of Chinese toad venom derived from the parotid secretion of *Bufo bufo gargarizans,* using chromatography both for detection and for preparation of material for subsequent bioassay(9).

The work to be described is the result of attempts to determine the presence of norepinephrine in a preparation of epinephrine derived from the parotid glands of the giant tropical toad *Bufo agua.* The epinephrine had been prepared by Abel in 1914, presumably according to the method described in the paper of Abel and Macht(1). It had been kept in this laboratory in a tightly-stoppered weighing bottle since the time of its preparation, and was in the form of a brown powder. Utilizing the modified Folin method of Barker, Eastland, and Evers(10), 1 μg of the toad "epinephrine" was found to give the same color intensity as 0.93 μg of pure epinephrine.[‡] Since pure norepinephrine produces less intense color, mg per mg, than epinephrine, its presence in the extract would make the percentage of catechols higher than that indicated by the above figures.

Paper chromatography. The technic utilized was essentially that of James(11). Epinephrine, norepinephrine, and the material under investigation were dissolved in acid alcohol and applied to Whatman No. 1 filter paper along a line 1½ inches from the bottom of the sheet. A cylinder was then formed by approximating two edges with cellulose tape as described by Crawford and Outschoorn (12). The paper cylinder was then set in a tall glass jar containing a layer of phenol saturated with water at its bottom, the top of the jar covered to maintain saturation of the atmosphere, and the phenol allowed to ascend by capillary attraction for 16-20 hours. At the end of this time, the paper was removed, dried at room temperature, and developed with 0.44% potassium ferricyanide in a phosphate buffer of pH 7.8. The epinephrine standard was found to have an R_F of 0.49 to 0.58 and to yield a pink-red color which faded to a yellow-brown. The norepinephrine standard had an R_F of 0.27 to 0.33, and produced a lavender spot which faded to a chocolate-brown. The toad "epinephrine" yielded two distinct spots—a large one with an R_F of 0.52 to 0.56 and a much smaller one with an R_F of 0.32 to 0.33. Not only did the spots correspond in location to those produced by the pure standards, but their colors were identical. Quantitative assay was attempted by a comparison of the sizes of spots produced by varying mixtures of epinephrine and norepinephrine with those produced by samples of the toad "epinephrine." By this crude bracketing technic, the "epinephrine" was found to contain between 2 and 5% of norepinephrine (as base).

Chemical determinations. The Auerbach and Angell method(13) was employed with one modification. It was found that identical amounts of norepinephrine produced different intensities of color, depending on the amount of epinephrine present in the sample. Therefore, 3 standard curves were run, determining the color intensity produced by 0.05 to 0.2 mg norepinephrine in the presence of 2 and 4 mg

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‡ Synthetic l-norepinephrine and l-epinephrine, in the form of the bitartrates, were used as standards throughout this work. The l-norepinephrine contained less than 0.5% of d-norepinephrine. The l-epinephrine contained less than 0.1% of d-epinephrine, and less than 0.1% norepinephrine. We are indebted to Dr. John C. Seed of Sterling-Winthrop Research Institute both for a generous supply of these compounds, and for the above data on their purity.

epinephrine as well as the 1 mg recommended by the original authors. Samples of the toad "epinephrine" were then run through, in amounts of 1, 2 and 4 mg total base. The readings in each instance were read against blanks run with equivalent amounts of epinephrine, and the readings translated into the amount of norepinephrine present by use of the appropriate standard curve. The results obtained in this manner checked very well, with the percentage of norepinephrine ranging from 4.9 to 5.7%.

Discussion. The chromatographs clearly indicate the presence of at least two separate components in the toad "epinephrine" of Abel and Macht. These components migrate at rates identical with those of epinephrine and norepinephrine, and yield the same color reactions. In addition, the chemical studies disclose the presence of a compound which behaves like norepinephrine. The percentages of norepinephrine obtained with these two different technics are in good agreement. Further, Lee and Chen, using a similar toad preparation, have obtained effects on the blood pressure and nictitating membrane of the cat, with material eluted from the "norepinephrine area" of large scale paper chromatographs, that duplicate those produced by pure l-norepinephrine. Their estimate of 1-2% norepinephrine content is of the same order of magnitude as our figures. These data complement each other and strongly suggest that the second component is, indeed, norepinephrine.

It is of interest that Fischer and LeComte detected 0.5 μg norepinephrine per 10 μg of epinephrine in one of their gland preparations, and that their results with the cocaine-sensitized nictitating membrane were compatible with the presence of small amounts of norepinephrine in all of their extracts. These somewhat equivocal findings and the inability of Bacq and LeComte to demonstrate norepinephrine are easily explained by the small quantities of norepinephrine which seem to be present in toad secretions. Neither bioassay nor most of the currently available chemical technics could be expected to reveal clearly the presence of percentages of norepinephrine of 5% or less of the catechol content. As Lee and Chen point out, these problems are magnified by the use of crude secretion. It is suggested that in investigations of the epinephrine-norepinephrine content of biological material chromatographic technics should be considered if cruder methods do not reveal the presence of both amines.

The existence of norepinephrine in toad secretions demonstrates the ability of these animals to form the amine. This raises the possibility that other tissues of the toad, including those of the nervous system, may possess this ability, and that norepinephrine may play an important role in the normal physiology of these animals, perhaps analogous to that suggested for higher animals (14).

Summary. Chemical and chromatographic studies have revealed the presence of a substance, possessing characteristics of norepinephrine, in a preparation of "epinephrine" extracted from the parotid secretion of *Bufo agua*. This substance was present in amounts of 2-5%.

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Protective Action of N-Allylnormorphine Against Demerol.* (19250)

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Hart and McCawley(1) and Unna(2) showed that N-allylnormorphine would protect mice against the respiratory depressant action of morphine. This observation was later extended by Huggins, Glass, and Bryan (3) who demonstrated in dogs that N-allylnormorphine protected against lethal amounts of codeine (methylmorphine), Dilaudid (dehydromorphinone), Metapon (methyldihydromorphine), and Methadon (6-dimethylamino-4, 4-diphenyl-3-heptanone hydrochloride). However, no protective action was evident against Demerol (ethyl 1-methyl-4-phenylisonipecotate hydrochloride). In their experiments the dogs had previously received sodium barbital, and it seemed possible that a protective action of N-allylnormorphine against Demerol might have been present but obscured by the barbital, although this was not true for the lethal doses of the other drugs tested. This point was considered worth further investigation because of the extensive use of Demerol and the potentiality of N-allylnormorphine as an antagonist to lethal amounts of many of the other narcotics. It was tested by determining the effect of N-allylnormorphine on mice that had received an LD 50 of Demerol.

Method. All injections were made subcutaneously, using the hydrochloride salts of Demerol and N-allylnormorphine. Gaddum's method(4) was used for determining the LD 50 of Demerol, which for this strain† of mice was found to be 188.3 mg/kg, but for convenience of administration 190 mg/kg was used. The experimental animals were then given the LD 50 of Demerol followed within one minute by varying amounts of N-allylnormorphine. Subsequently trials were made in which the doses of the two drugs were kept constant but the time between their administration lengthened.

Results. N-allylnormorphine in either 10 or 20 mg/kg doses, if given within one minute after the Demerol, reduced the mortality to 7 and 8%, respectively (Table I). Amounts less than 10 mg/kg were not so effective although 0.25 mg/kg reduced the mortality to 23%. If the time of injection of N-allylnormorphine was delayed for 5 minutes after the administration of Demerol, it provided no protection (Table II).

Mice receiving N-allylnormorphine after Demerol were observed to have convulsions in only 14% of the trials as opposed to 66% in animals receiving Demerol alone. Examination of Table III will show that of the animals that had convulsions about 50% died, regardless of which treatment they received. The average length of time between the injection of Demerol and the time of the first convulsion was 29 minutes in N-allylnormorphine treated animals as opposed to 13 minutes for animals

TABLE I. Data on Mice Receiving an LD_{50} Dose of Demerol Followed Within One Min by Different Amounts of N-allylnormorphine.

No. of mice	N-allyl-normorphine, mg/kg	No. of mice surviving	No. dying	% dying
63	20	58	5	8
28	10	26	2	7
28	5	23	5	18
28	1	18	10	35
38	.5	30	8	21
30	.25	23	7	23

TABLE II. Data on Mice Given an LD_{50} Dose of Demerol Followed at Varying Intervals of Time by 20 mg/kg of N-allylnormorphine.

Time between admin. of Demerol and inj. of N-allyl-normorphine, min	No. living	No. dying	% dying
1 or less	58	5	8
5	9	10	53
10	13	6	32
15	8	10	55

* We wish to express our appreciation to Merck and Co., Inc., for the N-allylnormorphine.

† Test animals were Harlan's Swiss albino mice, males, avg wt 19.1 g.

TABLE III. Relationship Between Treatment Given, *i.e.* Demerol Alone in Varying Amounts or Demerol and N-allylnormorphine, and % of Mice Having Convulsions. Time of onset of first convulsion.

Demerol, mg/kg	% having convulsions	% mice showing convulsions who died during exp. period	Avg time between inj. of Demerol and 1st convulsion, min	Time between inj. and 1st convulsion, min
175	66	65	18	2-65
200	75	50	13	2-66
190, followed within 1 min by 20 N-allylnormorphine	14	60	29	5-93

treated with 200 mg/kg of Demerol and 18 minutes for those receiving 175 mg/kg of Demerol. There was a wide range of time intervals before the onset of the first convulsion, but in some mice convulsions developed within 2 minutes after the injection of Demerol.

Discussion. Contrary to the findings of Huggins, Glass, and Bryan(3) using dogs, N-allylnormorphine does have a protective action against Demerol when tested on mice. There would seem to be at least two possible explanations for their failure to find this effect. First, barbitalization of their dogs may have obscured the action of N-allylnormorphine, although this did not occur with the other narcotics they tested. The second possibility is that the very rapid action of Demerol in mice, and perhaps in dogs, necessitates the immediate use of N-allylnormorphine if any reduction in mortality is to be effected. For example, if N-allylnormorphine is given 5 minutes after the injection of Demerol, the number of mice dying approaches 50%, while Unna(2) found protection against an LD 50 of morphine for as long as 15 minutes after its injection. The rapidity with which Demerol may act is also indicated by the onset of convulsion within 2 minutes after its subcutaneous injection in some of the mice.

Summary. (1) N-allylnormorphine in amounts of 10 mg/kg or more, if given within one minute after an LD 50 of Demerol, provides significant protection in mice. (2) If N-allylnormorphine is given 5 minutes after the LD 50 of Demerol, it provides little protection even if 20 mg/kg is given.

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Nutrition of Animal Cells in Tissue Culture. VI. Low Toxicity of Barium.*† (19251)

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During studies on the development of a synthetic medium for animal cells in tissue culture(1-4), it was observed that small

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amounts of certain heavy metals exerted marked effects on cell survival and multiplication. Iron, *e.g.*, was found to be beneficial(1) whereas cobalt was somewhat inhibiting(4). Because several ingredients of the synthetic mixture were obtained commercially as barium salts, and were converted to sodium salts before use, it was thought desirable to determine the degree of toxicity of barium and the barium content of natural and synthetic media.

Materials and methods. Strain cultures of fibroblast-like spindle cells (hereafter referred to as *fibroblasts*) were derived from the leg muscle of 11-day-old chick embryos and maintained in Carrel D-3.5 flasks in a coagulum consisting of fowl plasma, chick embryo extract(5) and balanced salt solution(6) for at least 5 passages (weeks) before use. Roller-tube cultures of chick mesenchyme tissues were prepared from the leg muscle of 11-day-old embryos and maintained in synthetic Mixture 199(1-3). Full details of the various assay procedures have already been described (1,3,4). A stock solution of barium acetate (reagent grade), containing 10 mg barium per ml, was prepared in glass-distilled water, sterilized by autoclaving, and diluted to the desired levels shortly before use. Barium was added to Mixture 199 in place of an equal volume of water, and to the plasma cultures in place of equal volumes of balanced salt solution. The barium content of natural and synthetic media was determined by the mixed-crystal method of Yagoda(7). Before making the assays, the samples (10 ml) were repeatedly ashed with concentrated nitric acid, and lead and iron were removed by successive extraction with chloroform solutions of diphenylthiocarbazone and 8-hydroxyquinoline.

Experiments and results. A. Effect of barium on fibroblasts cultivated in a medium containing plasma and embryo extract. Barium was added to fibroblast cultures at final concentrations ranging from 0.01 to 1000 μg per ml. Below 1 μg per ml there was no appreciable effect, but a progressive inhibition was observed as the barium concentration was increased from 1 to 1000 μg per ml (Table I). Because the barium precipitated at 100 and 1000 μg per ml, higher levels were not tested.

TABLE I. Effect of Barium on Area of Outgrowth of Representative Cultures of Chick Fibroblasts Propagated in Carrel Flasks in a Medium Containing Fowl Plasma and Chick Embryo Extract.

Barium conc. (μg per ml)	No. of cultures	Avg inhibition, %*
.01	11	10†
.1	10	10†
1	10	20
10	11	22
100	7	35
1000	..	39

* Inhibition was calculated as percentage of the area of outgrowth of sister control cultures not exposed to barium.

† Inhibitions of 10%, or less, are not considered significant.

TABLE II. Effect of Barium on Length of Survival of Chick Embryo Mesenchyme Tissues Cultivated in Roller Tubes in Synthetic Mixture 199.

Barium conc. (μg per ml)	No. of cultures*	Avg survival, days
0†	12	37
.1	12	33
1	6‡	29
10	14	31
100	14	36
1000	13	27

* Data summarized from results of 3 typical experiments.

† Mixture 199.

‡ Several cultures in this group were lost through bacterial contamination.

B. Effect of barium on chick embryo mesenchyme tissues cultivated in a synthetic medium. In roller-tube cultures maintained in synthetic Mixture 199, barium was tested at levels ranging from 0.1 to 1000 μg per ml (Table II). In each of three separate experiments, no consistent effect upon the survival of the cultures was noted even at barium concentrations that inhibited, very appreciably, the outgrowth of fibroblasts cultivated in plasma and embryo extract. In general, however, cultures exposed to barium did not appear quite as healthy as the control cultures. As in the previous experiments, it was difficult to keep the barium in solution in Mixture 199, and the highest levels tested (100 and 1000 μg per ml) were very turbid.

C. Barium content of natural and synthetic media. Barium determinations were made on 8 samples of Mixture 199, on 2 samples each of hexose diphosphate and adenosinetriphos-

phate (metabolites that had been converted from barium to sodium salts) and on 5 samples each of fowl plasma and chick embryo extract. In all instances, the barium content was less than 5 μg per 10 ml, an amount too low to cause an appreciable effect upon the cultures. Although the assay method used is not extremely sensitive, the quantitative recoveries obtained when known amounts of barium were added to the samples showed that the results were reproducible.

Discussion. The results of the present experiments, based on more than 300 cultures, indicate that barium, in moderate amounts, is slightly toxic to tissues cultivated in flasks in naturally-occurring media but is relatively non-toxic to roller-tube cultures supplied with synthetic media. Verne and Sannié(8) and Hogue(9) have previously reported that barium is only slightly toxic to short-term hanging-drop cultures in natural media.

The observation that relatively high concentrations of barium do not shorten the period of survival of tissues cultivated in synthetic medium is somewhat surprising since these same concentrations reduce the area of outgrowth of fibroblasts in natural media. In a previous publication from this laboratory (4) it was shown that small amounts of cobalt are extremely toxic to tissues cultivated in natural media, but are non-toxic to tissues maintained in synthetic media. This lack of toxicity of cobalt was found to be due to the protective action of the L-histidine contained in the synthetic medium. No explanation for the lack of toxicity of barium under similar conditions has yet been obtained.

Many metabolites of nutritional interest are available commercially only as barium salts. The low solubility of these compounds makes it important to convert them to soluble forms before adding them to the culture media. In most instances, the corresponding sodium salts have suitable properties. The conversion is conveniently carried out by dissolving the barium compound in dilute mineral acid and adding saturated sodium sulfate until no further precipitate forms. The barium sulfate precipitate is centrifuged off and the pH of the supernatant adjusted to neutrality with dilute sodium hydroxide. Because barium sulfate has a low solubility product, the conversion is quantitative and there is no carry-over of barium. The effectiveness of this method is confirmed by chemical assays on the media.

Summary. Relatively high concentrations of barium (1 to 1000 μg per ml) reduce the area of outgrowth of chick fibroblasts propagated in a medium containing fowl plasma and chick embryo extract. But these same concentrations of barium do not shorten the period of survival of chick embryo mesenchyme tissues maintained in a synthetic medium. Chemical analyses showed the barium content of the natural and synthetic media that were used to be less than 5 μg per 10 ml.

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Plasma Cholesterol of Roosters under Various Conditions. (19252)

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Gofman *et al.*(1,2) and Duff and Payne(3) suggest that the physical state in which cholesterol occurs in the plasma may be an important factor in the development of atherosclerosis. Previous reports from our laboratory(4,5) have shown that only a relatively small percentage of the total cholesterol of lyophilized normal human serum can be extracted by cold chloroform in a 3-hour period. It has also been shown that in some cases the amount of this fraction may be considerably increased even though the total cholesterol may be within normal limits(6). Its concentration is invariably high in rabbits rendered hypercholesterolemic from the administration of high cholesterol diets as well as in nephrotic patients with hypercholesterolemia (4). It was suggested that the degree of extractability under these conditions was influenced by the physical state of the cholesterol present.

Since atherosclerosis can be readily produced in roosters by addition of cholesterol to their diet(7) or by administration of estrogenic substances(8,9) it was decided to study changes in the concentration of this "readily extractable" cholesterol fraction following administration of estrogenic substances or of a high cholesterol diet to 3-month-old cockerels. Concomitant studies were carried out on the effect on the cholesterol fractions of the addition of lecithin or of neutral fat to the basal diet.

Methods. Rockland laying mash was used as the basal diet in all cases. Its fat content is about 3.5%. Two estrogenic preparations were employed, diethylstilbestrol and Dianisylhexane.* The diethylstilbestrol was implanted subcutaneously in pellet form.† Each pellet contained 12 mg of the estrogenic material and one pellet was implanted at a time. The Dianisylhexane, which was used at a level of 20 and 40 mg per kilo of diet, was dissolved in Mazola oil and added to the basal diet. The Mazola oil constituted 5% of the finished diet. The cholesterol was dissolved in chloroform, mixed with sufficient Mazola oil to make up 10% of the finished diet, and the whole thoroughly mixed with the laying mash. The chloroform was allowed to evaporate before the diet was given to the experimental animals. Blood was obtained from the alar vein, heparin being used as an anticoagulant. Serum was analyzed in a few cases and the results were in good agreement with those obtained on heparinized plasma from the same blood. The analytical procedures have been described(4).

Results. Typical results obtained with diethylstilbestrol are shown in Table I. It will be seen that its administration caused a marked increase in the concentration of the "readily extractable" fraction, as well as in total cholesterol. Animals receiving Dianisylhexane showed the same general changes but the individual variations were much greater. Some of the animals were very susceptible to the drug while others were resistant. It is possible that this difference in susceptibility may have been due to variations in the absorption of the estrogenic material from the gastrointestinal tract. The effect of cholesterol feeding on the various fractions is shown in Table II. It will be seen the concentration of the "readily extractable" cholesterol as well as that of the total cholesterol showed a decided increase during the cholesterol feeding and dropped to normal within a relatively short period of time after discontinuation of the cholesterol administration. A comparison of the data on the individual animals with those obtained when diethylstilbestrol was administered showed that, for moderate cholesterol concentrations, the increase in the "readily extractable" cholesterol was much

* Dianisylhexane was supplied by White Laboratories, Inc., Newark, N. J., through the courtesy of Dr. C. W. Sondern.

† Capette pellets, manufactured by Wick and Fry, Inc., Cumberland, Ind.

TABLE I. Effect of Diethylstilbesterol Administration on Plasma Cholesterol of Roosters.

No. of animals	Avg concentration per 100 ml			Remarks
	R. E. chol., mg	Total chol., mg	N. F. + chol., mg	
11	8 (5-12)	87 (34-127)	273 (172-416)	Control animals on laying mash
5	168 (24-413)	244 (157-445)	1173 (553-2415)	41 days after starting diethylstilbesterol inj.
	249 (83-421)	283 (114-417)	2240 (527-5210)	74 days after starting diethylstilbesterol inj.

Range in parenthesis. R. E. chol. = readily extractable cholesterol. N. F. + chol. = neutral fat + cholesterol.

TABLE II. Effect of Cholesterol Feeding on Plasma Cholesterol of 5 Roosters.

Avg concentration per 100 ml			Remarks
R. E. chol., mg	Total chol., mg	N. F. + chol., mg	
200 (18-536)	344 (200-598)	600 (377-960)	After 13-25 days on .5% followed by 8 days on 1% cholesterol diet
172 (41-689)	368 (223-701)	785 (557-1345)	After 22 days on 1% cholesterol diet
213 (26-772)	448 (236-829)	856 (356-1783)	" 92 " " " " "
8 (6-12)	72 (16-104)	218 (131-303)	25 days after cessation of cholesterol feeding

Range in parenthesis. R. E. chol. = readily extractable cholesterol. N. F. + chol. = neutral fat + cholesterol.

TABLE III. Effect of Addition of Either Neutral Fat or Lecithin to Diet on Plasma Cholesterol of Roosters.

No. of animals	Avg concentration per 100 ml			Remarks
	R. E. chol., mg	Total chol., mg	N. F. + chol., mg	
7	8 (5-10)	84 (34-127)	285 (172-416)	Control period, laying mash diet
	43 (7-115)	117 (36-161)	277 (54-384)	After 27 days on 20% Crisco diet
	27 (9-60)	81 (23-131)	209 (86-306)	" 40 " " 20% " "
	9 (6-13)	67 (31-120)	263 (131-356)	" 39 " " 10% " "
5	11 (5-20)	116 (86-129)	342 (273-452)	" 22 " " 10% " "
	10 (5-15)	137 (116-160)	367 (231-475)	" 68 " " 10% " "
	6 (3-8)	67 (32-115)	132 (83-201)	After low-fat diet 10-13 days
	5 (3-7)	77 (35-113)	109 (59-154)	" " " " 28 days
	11 (7-15)	116 (91-154)	315 (270-364)	After 13 days on 10% Crisco diet
7	5 (2-8)	67 (37-85)	171 (107-208)	" 28 " " 10% lecithin diet
	5 (3-6)	41 (24-62)	121 (53-255)	" 83 " " " " "

Range in parenthesis. R. E. chol. = readily extractable cholesterol. N. F. + chol. = neutral fat + cholesterol.

less than when the total cholesterol was raised to the same level by the administration of estrogenic substances. However, when the cholesterol content of the plasma was high (about 700 mg or above per 100 ml) in the cholesterol fed animals, nearly all of it was present in the "readily extractable" form. A similar condition was found in the plasma of the animals receiving estrogenic substances at a much lower total cholesterol level.

There is evidence that, in order to lower the plasma cholesterol of human subjects by dietary means, it is usually necessary to reduce to a low value the amount of fat in the diet as well as its cholesterol content. Consequently, it was considered of interest to determine if, in the cockerel, the level of fat in the diet would influence the concentration of either the total cholesterol or the "readily extractable" fraction. The experimental results are shown in Table III. It will be seen that when the diet contained 20% Crisco, making a total fat content of approximately 23.5%, the concentration of the "readily extractable" fraction showed on the whole a definite rise while that of total cholesterol and of neutral fat showed no consistent change. Only one of the birds failed to show a distinct rise in

the "readily extractable" fraction during the experimental period. When the diet contained 10% Crisco the concentration of the "readily extractable" cholesterol fraction closely approximated the levels found in the cockerels on the basal diet itself. When the basal diet, repeatedly extracted with carbon tetrachloride to remove fat and then supplemented with vitamins A, D, E, thiamine, riboflavin, pyridoxine, niacin and calcium pantothenate was fed, the concentration of the "readily extractable" fraction showed a uniform drop. Similarly low values were obtained when the basal diet was supplemented with sufficient soybean lecithin‡ to constitute 10% of the diet. It will be seen that in this case the total cholesterol concentration dropped to a very low value by the time the birds were on the diet for 83 days. This effect of the soybean lecithin is of considerable interest in view of the fact that Steiner and Domanski(10), Gross and Kesten(11), Adlersberg and Sobotka(12) obtained a drop in the serum cholesterol of patients with various pathological conditions following oral administration of a soybean lecithin preparation.

Summary. Subcutaneous administration of diethylstilbestrol to cockerels caused a marked increase in the concentration of the "readily extractable" cholesterol of the plasma as well as in total cholesterol and neutral fat. Cholesterol feeding exerted a similar effect, but at moderately elevated plasma cholesterol levels the concentration of the "readily extractable" fraction was not as great as when the total cholesterol was raised by the administration of estrogenic substances. However, when the total cholesterol was high, about 700 mg %, practically all of it was present in the "readily extractable" form. Roosters receiving a high fat diet for several weeks usually showed a definite increase in the "readily extractable" fraction without a consistent increase in total cholesterol. When the diet contained 10% soybean lecithin the concentrations of both the total cholesterol and of the "readily extractable" fraction dropped below those found in animals on the basal diet.

‡ A dry granular material obtained from Fries Bros., Inc., N. Y. City.

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